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FOREWORD

The Council for Scientific and Industrial Research, with the approval and co-operation of the Australian National Research Council, ventures to launch this new periodical, the Australian Journal of Scientific Research.

The issue will at first be in two series, covering papers relating to physical sciences and biological sciences, respectively.

The aim is to encourage scientific endeavour in the Commonwealth, and it is believed that both quality and quantity of material available for publication will justify the step now taken.



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THE NATURE OF REACTION WOOD

I. THE STRUCTURE AND PROPERTIES OF TENSION WOOD FIBRES

By A. B. WARDROP* and H. E. DADSWELL*

(Plates 1-4)

[Accepted for Publication January 14, 1948]

Summary

An examination has been made of the cell wall structure of tension wood fibres isolated from several Australian species including *Eucalyptus regnans* F.v.M., *Eucalyptus gigantea* Hook. f., *Nothofagus cunninghamii* Oerst. and *Acacia melanoxylon* R. Br.

It has been demonstrated that the so-called tertiary or gelatinous layers of these fibres are unlignified and consist almost entirely of a very highly oriented form of wood cellulose.

X-ray, optical, and visual evidence suggests that the secondary wall of tension wood fibres of *Eucalyptus regnans* F.v.M. consists of three layers of different micellar orientation: an outer layer in which the micelles are oriented at 40° to the longitudinal fibre axis, a middle layer where the angle of micellar orientation is 18° , and a broad, unlignified, inner, tertiary layer in which the micelles are oriented at approximately 5° to the longitudinal fibre axis.

Reference has been made to the possible relationship between the abnormal properties of tension wood and the nature of the cell wall: it has been suggested that the lack of lignin in the cell wall may account for the high tensile strength and low compressive strength of tension wood.

No satisfactory explanation of the high longitudinal shrinkage of tension wood has been forthcoming.

Slip planes and minute compression failures in tension wood have been discussed in relation to Chow's claim (1946) that incipient tension failures are present in tension wood fibres.

I. INTRODUCTION

The structure of tension wood fibres is of considerable academic and practical interest, both in relation to considerations of the stimuli which produce them, and to studies of the influence of fibre structure on the properties of the wood as a whole. As is well known, the chief abnormal properties of tension wood lie in its unusually high longitudinal shrinkage, its high tensile strength, and its low compressive strength.

Until recently, perhaps the most detailed study of fibre structure in tension wood was that of Munch (1938). This investigator has pointed out that in tension wood fibres, in addition to the lignified primary and secondary layers,

*Division of Forest Products, C.S.I.R.

there is an additional "tertiary" layer which is relatively unlignified. It is this tertiary layer* which has often been described as the "gelatinous" layer and has frequently led to tension wood fibres being described as "gelatinous fibres" (Rendle 1937). More recently Chow (1946) and Preston and Ranganathan (1947) have described various aspects of the structure of tension wood, and in relation to these studies, the following account of the structure of tension wood fibres from various Australian species should prove of interest.

Before describing the properties of tension wood fibres in detail, it is convenient to consider as a basis of comparison, the structure of normal wood fibres. The most important cell wall constituent is cellulose consisting of long-chain molecules built up from anhydroglucopyranose residues, and measuring 1,000-3,000 Å (1Å = 10^{-8} cm.) in length. These molecules, over certain regions of their length, are perfectly aligned with respect to one another, so as to form a type of crystal lattice. These regions of high molecular orientation in the cellulose are known as micelles and measure, so far as can be ascertained from X-ray data, about 50Å by 60Å by at least 600Å. Between the micelles are cellulose chains in more or less random orientation and also other cell wall constituents such as lignin, hemicelluloses, and mineral matter. Because of the perfect molecular alignment in the micellar regions of cellulose, the cell wall exhibits certain crystalline properties such as optical anisotropy. In a typical fibre or tracheid the outermost layer of the cell wall is the primary wall formed at cell division, and inside this is the much thicker secondary wall. If a cross section of a fibre or tracheid is viewed between crossed nicols, three distinct layers of different optical properties can be identified in the cell wall: a brighter outer and inner layer, together with a dark middle layer (Plate 1, Fig. 1). The primary wall also appears bright between crossed nicols but cannot always be distinguished from the much brighter outer layer of the secondary wall. This difference in optical properties of the various cell wall layers has been interpreted in terms of differences in micellar arrangement within them (Kerr and Bailey 1934; Preston 1934, 1946). It has been established that in the primary wall the micelles are oriented almost transversely to the longitudinal fibre axis, while in the middle layer of the secondary wall they are oriented at only a small angle to the longitudinal fibre axis. In the outer and inner layers of the secondary wall the micelles are, according to Kerr and Bailey (1934), oriented almost transversely to the longitudinal fibre axis, and it is because of this, according to these investigators, that the layers are strongly birefringent between crossed nicols. However, Preston (1934, 1946), working exclusively on conifer tracheids, has ascribed the optical properties of the various cell wall layers to different types of micellar angular dispersion about the spiral axis of the general cellulose chain direction, so that while the micellar angular dispersion differs in the three layers of the secondary wall, the inclination of the cellulose chains to the longitudinal cell axis is almost constant. Preston (1947) has also made a detailed

*Throughout this paper the inner gelatinous layer of the tension wood fibre will be referred to as the "tertiary layer."

study of the optical behaviour of the various cell wall layers and has concluded that, in the secondary wall, no layer exists with transversely oriented chains sufficiently extensive to affect materially the optical properties of the wall. However, from work since carried out by one of us (A.B.W.) in collaboration with Dr. R. D. Preston in the Botany Department at the University of Leeds, it has been established that both orientation and dispersion play a part in determining the optical heterogeneity of the cell wall.

For this purpose an examination was made of the changes in birefringence for the different wall layers in a series of sections cut at increasing angles to the radial longitudinal plane. It was shown that in the outer bright layer, illustrated in Plate 1, Figure 1, the micelles are oriented in a rather flat spiral with respect to the longitudinal cell axis, while in the dark layer the micellar orientation is in the form of a steep spiral (Wardrop and Preston 1947). In both the bright and dark layers, however, there exists considerable angular dispersion about the spiral direction, both in the plane of the wall and, in the case of the dark central layer, in a plane perpendicular to the wall surface as well. The dispersion, together with composition differences, gives rise to rather low values for the maximum birefringence in the various cell wall layers—these are of the order of 0.04 for the dark central layer and 0.02 for the bright outer layer (cf. ramie—0.06). This fact is of importance in determining the micellar orientation in the different cell wall layers. It will be clear, however, that the type of cell wall organization proposed by Kerr and Bailey (1934) is essentially the correct one, and their conception will be employed in the present paper (see Fig. 1A).

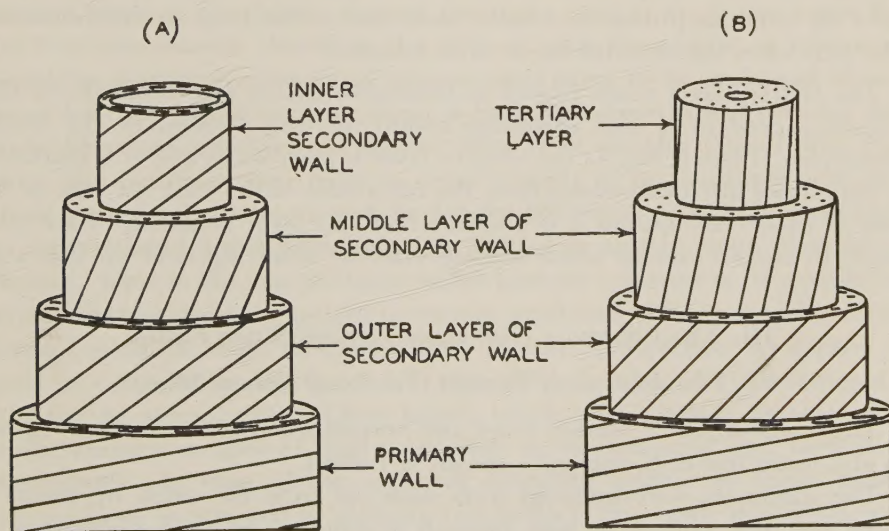


Fig. 1.—Diagrammatic representation of the structure of a normal wood fibre (A) and a tension wood fibre (B) of *Eucalyptus regnans* F.v.M. in which successive cell wall layers are considered partially removed.

In the investigations described below, it was necessary to establish the difference between the structure of the tension wood fibre and that of the normal fibre, especially as regards the relationship of the "tertiary" layer of Munch to the other cell wall layers. Also, in considering the abnormal physical properties of tension wood, it will be apparent that these will depend on three main factors: (i) The fine structure; (ii) the composition of the fibres; and (iii) the arrangement and proportion of the fibres and other tissue elements (rays and vessels) within the wood. It is with these factors, together with a consideration of the comparative cell wall structure of tension wood and normal wood fibres, that this paper is primarily concerned.

II. FORM AND THICKNESS OF THE TERTIARY LAYERS IN TENSION WOOD FIBRES

In the present investigation four species were examined:

Eucalyptus regnans F.v.M.

Eucalyptus gigantea Hook.f.

Nothofagus cunninghamii Oerst.

Acacia melanoxylon R.Br.

The tertiary layer is easily distinguished in cross sections of tension wood fibres by its gelatinous appearance and capacity to stain strongly with light green (Plate 1, Fig. 2) and to give a strong positive reaction with cellulose cytological reagents. The thickness of the tertiary layer varies greatly; all stages, from completely normal cells to those in which the lumen is almost completely filled, are observed. This variation in thickness could possibly be related to the duration of the stimulus producing tension wood and to the stage of differentiation of the cells when the stimulus began to be effective.

The tertiary layer when viewed in transverse section sometimes shows slits or cracks suggestive of high shrinkage within the cells themselves. In *Acacia melanoxylon* (Plate 1, Fig. 3) the tertiary layer is peculiar in that it is markedly convoluted and often withdrawn from the remainder of the wall. In view of the extent of these convolutions it is unlikely that this layer could ever have been directly in contact over its whole surface with the other layers of the cell wall.

III. THE CHEMICAL NATURE OF TENSION WOOD FIBRES

(a) *The Analysis of Tension Wood and Normal Wood*

The composition of tension wood and normal wood from the same annual ring of a young tree of *E. regnans* is shown in Table 1.

The specimens were removed from discs cut from the trunk by means of a band saw and ground to pass through a standard 60-mesh sieve prior to analysis. The analyses were carried out according to the standard methods of the Forest Products Division of the Council for Scientific and Industrial Research, Australia.

TABLE 1

THE COMPARISON OF NORMAL AND TENSION WOOD

	Normal Wood (%)	Tension Wood (%)
C and B cellulose	55.8	63.5
Xylan* (total)	18.3	11.5
Lignin	22.2	16.0
Alkali solubles	10.7	7.95
Total methoxyl	7.78	5.67
Apparent lignin	27.0	18.7
Xylan in C and B cellulose:		
As cellulose	20.6	9.87
As wood	11.5	4.27
Methoxyl in apparent lignin:		
As apparent lignin	21.4	20.3
As wood	5.71	3.74

*Furfural yield calculated as xylan.

These results are of interest in relation to the statement frequently made that tension wood is less lignified than normal wood, this being based on the apparently low lignin content of tension wood as shown in Table 1. In order to make such a comparison of lignin content, however, it is necessary to determine the relative amounts of lignin in a tension wood fibre compared with a normal wood fibre. This can be done approximately by considering the density of tension wood in comparison with normal wood in relation to the analytical data. Numerous determinations have shown that the densities of tension wood and normal wood in *Eucalyptus regnans* are in the ratio of 4 : 3, the actual figures for the basic density (based on the ratio of oven dry weight to volume when soaked to maximum volume) of tension wood being 40 lb./cu.ft. and of normal wood being 30 lb./cu.ft. Since there is little difference in size between tension wood fibres and normal wood fibres (Chow 1946; see also Plate 1, Fig. 2), and since the analytical data refer to equal weights of tension wood and normal wood, these data will, because of the density difference, cover only three-quarters as many tension wood fibres or parts thereof as normal wood fibres or parts thereof. Thus in the case of lignin which analysis indicates to be present to the extent of 16 per cent. by weight in tension wood and 22.2 per cent. by weight in normal wood, in order to make a fibre by fibre comparison the former value must be corrected by the factor 4/3, thus giving a value of 21.3 compared with 22.2 for the normal wood. These values, which are no longer percentages, represent relative weights of lignin per fibre in tension wood and normal wood respectively. In view of the apparent complete absence of lignin from the tertiary layer as revealed by staining (see below), this result would suggest that the extent of lignification of tension wood fibres and normal wood fibres is approximately the same prior to the development of the tertiary layer in the abnormal fibres (see Discussion).

Further consideration of the data in Table 1, with particular reference to the low xylan content of the Cross and Bevan cellulose in tension wood, and also to the high proportion of this resistant form of cellulose compared with normal wood, suggests that the ratio of crystalline cellulose to amorphous material may be higher in tension wood than in normal wood. Evidence which would add support to this possibility can, in fact, be obtained by the study of the comparative hydrolysis rates of tension wood and normal wood, and of the corresponding holocellulose fractions, using dilute acid (see Table 2). The values were obtained by measuring the loss in weight of the wood or of the holocellulose after hydrolysis for a specified period of time and expressing the result as percentage hydrolysis calculated on the initial weight.

TABLE 2

HYDROLYSIS OF TENSION WOOD AND NORMAL WOOD OF EUCALYPTUS REGNANS AND OF CORRESPONDING HOLOCELLULOSE FRACTIONS USING 2N HCl AT 100° C.

Time of Hydrolysis (min.)	Hydrolysis of Wood (%)		Hydrolysis of Holocellulose (%)	
	Normal	Tension	Normal	Tension
0	0.00	0.00	0.00	0.00
15	22.74	22.50	28.62	27.29
30	25.70	24.20	—	—
60	31.49	28.73	38.06	33.89
120	31.56	29.75	42.92	36.42

It will be noted that the extent of hydrolysis in a given time for tension wood and tension wood holocellulose is considerably less than that for the normal wood or its holocellulose fraction, and this is probably an indication of a higher degree of crystallinity in the tension wood cellulose and is consistent with the X-ray evidence (see below) and low xylan content of tension wood: all this evidence would indeed point to the conclusion that tension wood contains a very highly oriented form of wood cellulose.

(b) *The Lignin Distribution in Tension Wood Fibres*

The tertiary layer of tension wood fibres stains strongly with cellulose stains such as light green, while the remaining cell wall layers stain with lignin stains such as safranin. The tertiary layers stain strongly with iodine and sulphuric acid. They are also insoluble in 17.5 per cent. sodium hydroxide, indicating that they consist of chemically resistant cellulose which is relatively unligified. However, the most striking evidence of lignin distribution in tension wood fibres can be obtained by staining sections using the method of Coppick and Fowler (1939). This method is in fact a modified Tollens reaction; the section is first treated with chlorine water when reducing substances are formed from the lignified tissues and the section is then immersed in an aqueous solution of silver nitrate when silver is deposited in the lignified areas. That the tertiary layer

lacks lignin according to this stain is shown in Plate 1, Figure 4, which is a cross section of *Nothofagus cunninghamii* tension wood. Similar results were obtained with the other species used in this investigation. Thus it will be clear that the tertiary layer of tension wood fibres is almost entirely free of lignin, and this, taken in conjunction with the purely chemical evidence, suggests that the tertiary layer consists of a form of wood cellulose of exceptional purity.

(c) *The Distribution of Pectin in Tension Wood Fibres*

If tension wood was sectioned immediately after removal from the tree the cells stained strongly with ruthenium red (1 : 10,000 aqueous solution) which is reputedly a pectin stain. The staining reaction was localized entirely in the unligified tertiary layer while the lignified areas gave no reaction (Plate 2, Fig. 1). After digestion with dilute hydrochloric acid at 90°C. for 20 minutes, the sections no longer gave the reaction, presumably due to the removal of pectins or hemicelluloses (Plate 2, Fig. 2). The exact nature of this pectin fraction is difficult to characterize because a positive staining reaction was obtained even after extraction with water, 0.5 per cent. ammonium oxalate (90°C.) and 0.5 per cent. oxalic acid (90°C.), so that apparently considerable hydrolysis was necessary before the material was removed. If this material were pectic in nature (when presumably it would be in combination, possibly as the calcium complex) it is consistent with previous evidence that pectin cannot be demonstrated in any but unligified tissues of the plant (Onslow 1931).

IV. EVIDENCE REGARDING THE STRUCTURE OF TENSION WOOD FIBRES

(a) *The Crushing of Isolated Fibre Sections*

Herzog (1939) has employed the technique of crushing various textile fibres as a means of revealing their structure. However, this method, using whole fibres, did not reveal anything as to the layering of the cell wall. In the present work longitudinal sections of tension wood and normal wood were cut sufficiently thin ($6\ \mu$ - $8\ \mu$) so that in the section no whole fibre was included. The sections were then delignified until equivalent in composition to holocellulose. Treatment of the sections with 0.025 per cent. sodium hydroxide enabled the cells to be easily separated. The fibre sections so obtained were then treated with a 1 per cent. aqueous solution of congo red and crushed by rolling a glass rod over them. The fibres were photographed in green light (Plate 2, Figs. 3 and 4). It would appear from an examination of fibres treated in the above manner that at least two layers of different spiral angle are present in the tension wood fibres. In the outer layer of the cell wall (Plate 2, Fig. 3) the fibres are oriented at approximately 18° to the longitudinal fibre axis while in the inner layer (Plate 2, Fig. 4) the fibrillar orientation is at 15° to the longitudinal cell axis. In the normal wood fibre treated similarly, one prominent set of striations oriented at approximately 20° to the longitudinal fibre axis can be distinguished, together with two less prominent sets oriented at a large angle (see Plate 3,

Fig. 1). However, the striations at a large angle must be regarded with some reserve as an indication of micellar direction since they do not exhibit marked dichroic properties as do the striations oriented at 20° as well as both the layers in the tension wood fibre illustrated in Plate 2, Figures 3 and 4. The orientation of the outer layer of the cell wall in normal wood fibres is probably better indicated by the birefringence measurements of this layer when viewed in transverse section (see below).

It is not considered likely that this crushing technique would greatly alter the orientation of the cellulose within the fibre wall; since the major extinction position did not alter by more than 5° during the crushing process and treatment with alkali.

(b) Optical Properties of Tension Wood Fibres

The major extinction position was measured using longitudinal sections of tension wood cut at a thickness so as to include only one wall of the fibre and was found to be inclined at $5^\circ - 8^\circ$ to the longitudinal fibre axis compared with an inclination of $15^\circ - 20^\circ$ in normal wood fibres, thus indicating that, in tension wood fibres, the micelles are inclined at a considerably steeper angle than in normal wood fibres.

The difference in micellar orientation in the various cell wall layers of tension wood fibres is also revealed by the examination of thin cross sections between crossed nicol prisms (Plate 3, Figs. 3 and 4). Thus the tertiary layer appears dark, and this is consistent with the presence of a steep micellar spiral and presumably the same inner layer as that revealed by the crushing technique in Plate 2, Figure 4. Furthermore, the birefringence of the outer bright layer of the wall in *E. regnans* normal wood was 0.012 while the corresponding layer in tension wood (Plate 3, Fig. 4) was 0.009, indicating that this layer possesses a rather steeper spiral in tension wood than in normal wood (see Discussion).

(c) X-ray Examination of Tension Wood Fibres

Since the X-ray examination of tension wood fibres has been the subject of a detailed study by Preston and Ranganathan (1947), little need be said concerning the results presented here other than to compare them with those obtained by these investigators. The diffraction patterns of normal wood (Plate 4, Fig. 1) and tension wood (Plate 4, Fig. 2) were obtained from small blocks 1 mm. thick with the medullary rays parallel to the X-ray beam using Cu-K- α radiation and a specimen-film distance of 3 cm. The spread of the equatorial arcs reveals a micellar spiral angle of 23° in normal wood and of 18° in tension wood with respect to the longitudinal fibre axis. While this difference between the values for normal wood and tension wood is rather less than that observed by Preston and Ranganathan in the case of beech, the photographs otherwise show essentially similar features. The higher orientation of molecules with respect to one another in tension wood in comparison with normal wood is evidenced by the better definition of the X-ray diffraction arcs in the former case and is consistent with the chemical data presented in Tables 1 and 2.

(d) *External Striations on Tension Wood Fibres*

If longitudinal sections of tension wood are cut at a thickness of about $40\ \mu$ and delignified in the manner described in Section IV (a) of this paper, then subsequent treatment with dilute alkali enables the separation of whole tension wood fibres. These isolated fibres, when allowed to dry in air, develop marked longitudinal striations inclined at about 5° to the longitudinal fibre axis. A fibre so treated and viewed between crossed nicol prisms is shown in Plate 3, Figure 2. Presumably these striations arise from the crinkling of the outer cell wall layers by the shrinkage of the tertiary layer so that the striations represent the orientation of the tertiary layer in the fibre corresponding to the dark layer in Plate 3, Figure 4, and that illustrated in Plate 2, Figure 4.

V. DISCUSSION OF THE STRUCTURE OF NORMAL WOOD AND TENSION WOOD FIBRES

From the evidence presented above it will be clear that at least two cell wall layers of different micellar orientation exist in the wall of the tension wood fibre. Both of these, an outer layer oriented at approximately 18° and an inner layer oriented at approximately 5° to the longitudinal fibre axis, were demonstrated by the crushing technique applied to single cell walls. It is to be emphasized that these values represent maxima for the particular layers concerned, since some distortion as evidenced by the change of the major extinction position did occur during crushing and subsequent alkaline treatment. Further evidence of the existence of the layer oriented at 5° is seen in the value of the major extinction position ($5^\circ - 8^\circ$ to the longitudinal fibre axis) and also in the development of fine longitudinal striations on the fibre during drying. It is to be noted that the value obtained from the major extinction position is probably greater than that of the inner layer since the value measured would be intermediate between the cellulose chain directions of the two layers.

On the other hand, the existence of a layer oriented at 18° to the fibre axis, shown in Plate 2, Figure 3, is supported by the X-ray evidence of a similar spiral angle.*

Now both these layers are of a spiral angle steeper than that existing in the middle layer of the secondary wall of a normal wood fibre (about 23° in the case of *E. regnans*) and so presumably would appear dark when viewed between crossed nicols in transverse section; both these layers must then be included in the dark layer of Plate 3, Figure 4. The orientation of the layer appearing bright between crossed nicols can be calculated from the observed values of the birefringence of this layer when viewed in cross section (Section IV (b)) and assuming a maximum birefringence of 0.02 (see Introduction) which would indicate that the outer layer in normal wood is oriented at some 49° to the longitudinal fibre axis compared with 40° in tension wood. This is illustrated

*Actually the X-ray diagrams show only that the cellulose chains are dispersed, but do not distinguish between angular dispersion of the cellulose micelles and their arrangement in the cell as a spiral. The evidence is, however, strongly in favour of the latter view, and the angle measured by spread of the arcs is not far removed from the angle determined by other means.

in Figure 1. At the present time there is no evidence of any difference in the structure of the primary wall in tension wood as compared with normal wood although this point is under investigation. It is concluded that the outer and middle layers of the secondary wall in tension wood possess a steeper spiral orientation than do those of normal wood, and that the inner layer of the secondary wall, with its relatively flat spiral angle in normal wood, is replaced in tension wood with the very thick tertiary layer in which the micelles deviate by not more than 5° from the longitudinal fibre axis. These two structures are illustrated for comparison in Figure 1.

Since, in the recent work of Preston and Ranganathan (1947), only X-ray methods were employed, no consideration was given to the possible existence of more than one cellulose spiral existing in the cell wall. It is true that from the X-ray photographs there is no evidence of a spiral at 40° or 49° to the longitudinal fibre axis in either tension wood or normal wood, but, in view of the vastly different composition of the different wall layers, and especially as regards their lignin content and the extent of their micellar dispersion, it is to be expected that any contribution the outer layer may make to the X-ray diagram would be faint and diffuse (Wardrop and Preston 1947). However, apart from this, it will be appreciated that the angle of the micelles to the longitudinal fibre axis, as measured by the spread of the equatorial arcs, will be the greatest of any steep spirals present in the structure. Thus in the present case of tension wood, where two spirals of 5° and 18° are present, that of 18° will be indicated on the X-ray diagram and will effectively mask the smaller spread of the equatorial arcs due to the 5° spiral. In general, therefore, the structural interpretation here presented of the tension wood fibre is consistent with the X-ray, optical, and visual evidence.

The chemical composition of tension wood fibres also presents certain features of interest. Thus the analytical and hydrolysis data of Tables 1 and 2 suggest the presence of a highly crystalline form of cellulose. This fact, in conjunction with the staining reactions of tension wood fibres in cross section (Plate 1, Fig. 4) and the similarity of lignin content in tension wood fibres and normal wood fibres, suggests that the tertiary layer is virtually unlignified and presumably consists of this chemically resistant and crystalline cellulose fraction. This view is supported by the X-ray diagram of tension wood in which there is a marked absence of diffuse scattering by amorphous materials, leading to a generally clearer photograph than is obtained with normal wood.

VI. RELATION OF FIBRE STRUCTURE TO THE PROPERTIES OF TENSION WOOD

In view of the abnormal properties of tension wood it is of some interest to consider the possible relationship of these properties to the fibre structure of tension wood proposed above. Two such properties of particular interest are (i) the high longitudinal shrinkage of tension wood and (ii) the abnormal collapse of tension wood as observed in specimens of various eucalypts—in particular *Eucalyptus regnans*.

In the case of the former, Preston (1942) has examined the relation of shrinkage to the angle of inclination of the micelles to the longitudinal fibre axis while Frey-Wyssling (1940, 1943) has focused attention on the nature of the intercellular layer as the major factor involved. While it is probable that both factors are involved to some extent, it was considered of interest to investigate the shrinkage of the wood in comparison with that of the fibre. The high longitudinal shrinkage of tension wood is well established; for the present experiment, blocks from *Eucalyptus regnans* were used to make a comparison between the longitudinal shrinkage of tension wood and of normal wood, and it was again observed that the tension wood had considerably higher longitudinal shrinkage. From matched blocks, fibres were isolated by the holocellulose method and used for determining fibre shrinkage, if any. While the behaviour on drying of an isolated fibre is rather complicated, it can be stated that, in all drying experiments, the fibres from both normal wood and tension wood exhibit similar dimensional changes. Details of the work on fibre shrinkage will be published later. While the conditions of shrinkage of the blocks and that of the fibres are admittedly not the same, it might have been expected that some difference in the amount of shrinkage of the fibres would be detected, and in view of the absence of such evidence, it remains difficult to account for the abnormally high shrinkage of tension wood. Preston and Ranganathan (1947) have pointed to the difference in micellar spiral angle as a possible source of this difference, but admit that it is in itself insufficient to account for the shrinkage properties, and suggest the possibility that angular dispersion about the spiral direction may be an additional factor. While this may well be so it is also probable that the composition of the fibres may also be a pertinent factor. Thus the absence of lignin, which is less hydrophilic than cellulose, in the tertiary layer of tension wood may facilitate intermicellar movement (Preston 1943), and this is consistent with the high longitudinal shrinkage of the low lignin-containing textile fibres, but it is obvious that any conclusion on this problem in the absence of further data would be premature.

The extremely bad collapse of tension wood as observed in the various species of the genus *Eucalyptus* is of the type referred to as "non-recoverable." Neither the standard steaming treatment nor boiling in water induces recovery, and in this respect collapsed tension wood differs from the ordinary collapsed specimen. Here again the cell wall composition may be the responsible factor.

The suggestion in the recent paper by Chow (1946) that the spiral markings observed by him in tension wood fibres are responsible for the high longitudinal shrinkage of the wood demands some comment. These markings (see Plate VI, Figs. 3-5, and Plate VII of Chow's paper (1946) are described by the author as incipient tension failures and are regarded as cracks in the wall substance which, he suggests, close together during drying thus causing the fibre to contract in length. The form, behaviour in polarized light, and preferential staining properties of these deformations described by Chow suggest to the authors that these markings are, in fact, incipient slip planes and compression failures and

are similar to those described by them in tension wood and in a large number of other fibres (Dadswell and Wardrop 1946; Wardrop and Dadswell 1947) (see also Plate 3, Fig. 2). Further, Chow refers to the work of Jacobs (1945) which he claims demonstrates that considerable tensile stresses exist in the growing stem. However, this is only one of two hypotheses presented by Jacobs, the alternative hypothesis being that actually the whole growing stem may be in a state of compression, but the peripheral layers of the wood are in a state of tension relative to the wood nearer the centre of the stem so that the existence of features characteristic of compression in tension wood is understandable. Furthermore, it has been shown that slip planes and compression failures are very common in tension wood (Wardrop and Dadswell 1947), the distinction between these two kinds of deformation being that the minute compression failures extend over a number of fibres in a more or less straight line. It should also be noted that these features are more difficult to detect in whole fibres as used by Chow than in thin sections. Again, the common occurrence of the cell wall deformations in tension wood is quite understandable in terms of the explanation of the occurrence of dislocation marks in textile fibres given by Frey-Wyssling (1936). This investigator suggests that when a compressive stress is applied to a fibre the micelles tend to buckle because of their great length as compared with their lateral dimensions. Thus, in an unlignified fibre similar to that of tension wood, buckling could occur easily, giving rise to the formation of slip planes and minute compression failures. However, in a lignified fibre, lignin, packed between the micelles, resists the tendency of the micelles to buckle, and slip planes and minute compression failures are less common, so that, in heavily lignified cells such as the tracheids of compression wood, slip planes and minute compression failures are not found.

Such considerations also offer an immediate explanation of the extremely low compressive strength of tension wood as compared with normal wood. It will also be apparent that the tensile strength will not be greatly altered since the intermicellar lignin would be without influence on this property and the micellar spiral angle is only one factor in determining the tensile properties of timber. These also depend on the forces of intercellular adhesion, which presumably influence the toughness of the timber. In fact, there is some evidence that intercellular adhesion in tension wood is greater than in normal wood (Wardrop and Dadswell 1947). In conclusion, it will be apparent that while the strength properties of tension wood are readily understandable in terms of the submicroscopic morphology of the fibre, the shrinkage phenomena must, as yet, await further experimental data before a comprehensive explanation is attempted. The next step in this study must now be concerned with the physiological significance of the fibre structures described above in relation to growth regulating factors operating in the tree, and work is now in progress which should throw some light on this interesting problem, at the same time bringing it into the field of investigation of the forester and plant physiologist.

VII. ACKNOWLEDGMENTS

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The authors wish to acknowledge their indebtedness to Dr. W. E. Cohen, Division of Forest Products, C.S.I.R., for his advice in relation to the interpretation of the chemical data, and to Mr. A. J. Watson, of the same Division, for the chemical analyses given in Table 1. The authors are also indebted to Dr. R. D. Preston, of the Botany Department, University of Leeds, in whose laboratory the X-ray examinations and measurements of birefringence were made.

VIII. REFERENCES

- CHOW, K. Y. (1946).—*Forestry* 20: 62.
- COPPICK, S., and FOWLER, W. F. (1939).—*Paper Tr. J.* 109: T.S. 135.
- DADSWELL, H. E., and WARDROP, A. B. (1946).—*Nature* 158: 174.
- FREY-WYSSLING, A. (1936).—*Protoplasma* 25: 261.
- (1940).—*Holz Roh-Werkst.* 3: 349.
- (1943).—*Ibid.* 6: 197.
- HERZOG, A. (1939).—*Wbl. Papierfabr.* No. 22: 481.
- JACOBS, M. R. (1945).—*Commonw. For. Bur., Aust. Bull.* No. 28.
- KERR, T., and BAILEY, I. W. (1934).—*J. Arnold Arbor.* 15: 327.
- MUNCH, E. (1938).—*Flora* (N.S.) 32: 357-424.
- ONSLOW, M. W. (1931).—“Principles of Plant Biochemistry.” (Cambridge University Press.)
- PRESTON, R. D. (1934).—*Philos. Trans. B* 224: 131.
- (1942).—*Forestry* 16: 32.
- (1943).—*Proc. Roy. Soc. B* 130: 103.
- (1946).—*Ibid.* 133: 327.
- (1947).—*Ibid.* 134: 202.
- and RANGANATHAN, V. (1947).—*Forestry* 21: 92.
- RENDLE, B. J. (1937).—*Trop. Woods.* No. 52: 11.
- WARDROP, A. B., and DADSWELL, H. E. (1947).—*Coun. Sci. Industr. Res. Aust. Bull.* No. 221.
- and PRESTON, R. D. (1947).—*Nature* 160: 911.

EXPLANATION OF PLATES 1-4

PLATE 1

- Fig. 1.—Cross section of delignified normal wood fibres of *Nothofagus cunninghamii* Oerst. (somewhat swollen) viewed between crossed nicols, X 880.
- Fig. 2.—Cross section of *Eucalyptus gigantea* Hook. f. showing both tension wood fibres and normal wood fibres stained with safranin and light green, X 390.
- Fig. 3.—Cross section of *Acacia melanoxylon* R.Br. showing typical tension wood fibres stained with safranin and light green, X 390.
- Fig. 4.—A cross section of tension wood in *Nothofagus cunninghamii* Oerst. stained according to the method of Coppick and Fowler (1939). Note the preferential staining of the middle lamella zone and the lack of staining in the tertiary layer of the cell wall, X 650.

PLATE 2

- Fig. 1.—Cross section of freshly cut tension wood in *Eucalyptus gigantea* Hook. f. stained with ruthenium red. Note the staining on the cell wall layers but not in the middle lamella and adjacent layers. X 390.
- Fig. 2.—Cross section of tension wood in *Eucalyptus gigantea* similar to that shown in Figure 1 but boiled with 12 per cent. HCl before staining with ruthenium red. Note lack of staining. X 390.
- Fig. 3.—A tension wood fibre isolated from *E. regnans* F.v.M. that has been cut longitudinally and crushed after staining with congo red. Note angle of spiral in cell wall. X 880.
- Fig. 4.—Same fibre as illustrated in Figure 3 photographed at different focus showing angle of spiral of innermost tertiary layer. X 880.

PLATE 3

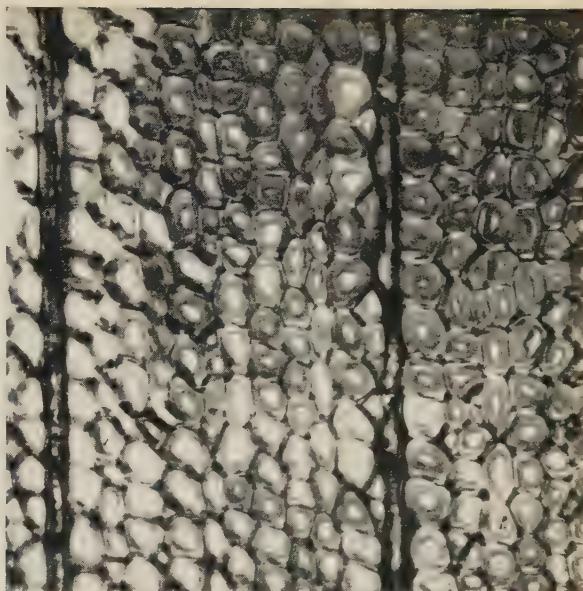
- Fig. 1.—Normal wood fibre from *E. regnans* F.v.M. cut longitudinally and crushed after staining with congo red. Note the definite striations oriented at a steep angle to the longitudinal fibre axis corresponding to the middle layer of the secondary wall. X 880.
- Fig. 2.—A tension wood fibre isolated from *E. regnans* F.v.M. viewed between crossed nicols. Note longitudinal striations at a very small angle to the fibre axis. X 390.
- Fig. 3.—A cross section of tension wood from *E. regnans* F.v.M. treated with Herzberg's stain. The tertiary layers of the cell walls stain a distinctive purple which is not observed in normal wood and is indicative of a high percentage of pure cellulose. X 880.
- Fig. 4.—The same section as illustrated in Figure 3 but viewed between crossed nicols. Note the absence of any indication of an inner layer as observed in normal wood fibres under similar conditions—compare Plate 1, Figure 1. X 880.

PLATE 4

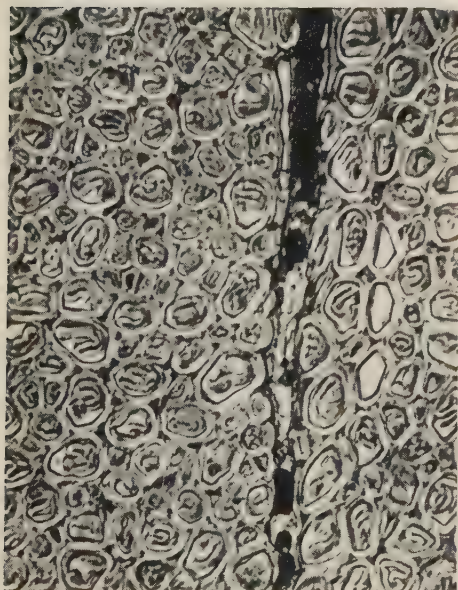
- Fig. 1.—X-ray diffraction photograph of normal wood of *E. regnans* F.v.M. Cu-K- α radiation specimen-film distance 3 cm.
- Fig. 2.—X-ray diffraction photograph of tension wood of *E. regnans* F.v.M. Note evidence of greater orientation. Cu-K- α radiation specimen-film distance 3 cm.



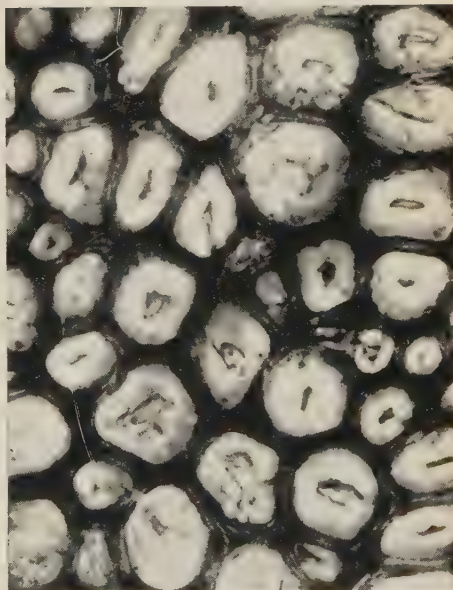
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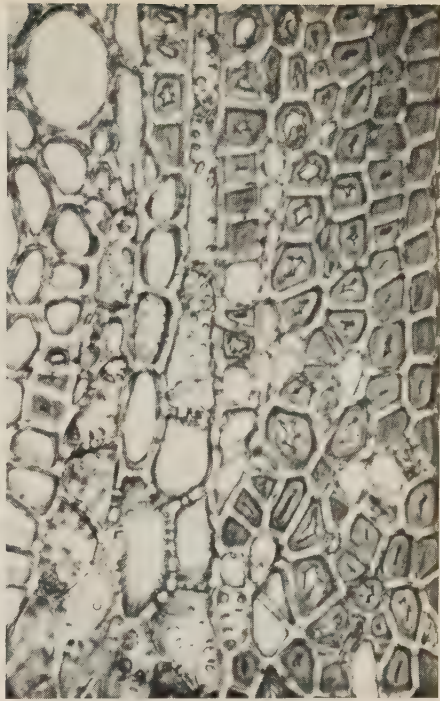
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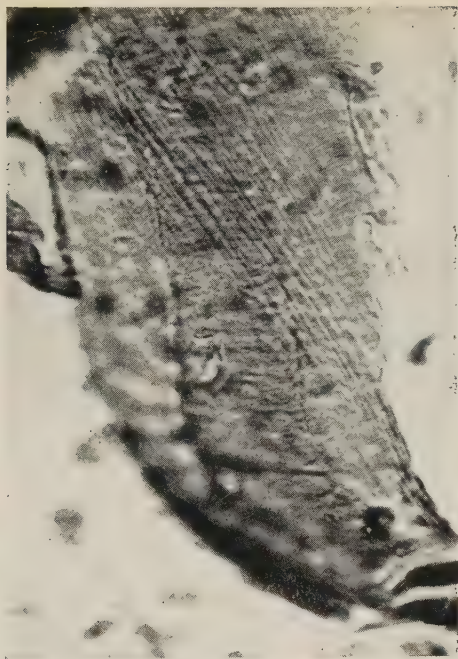
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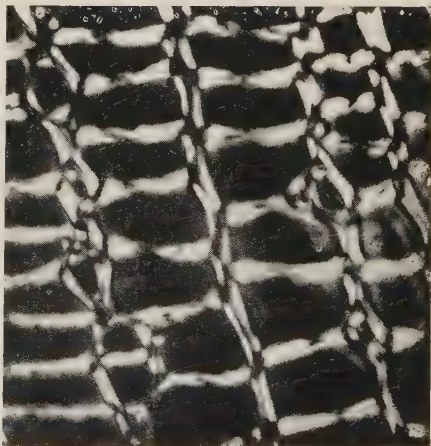
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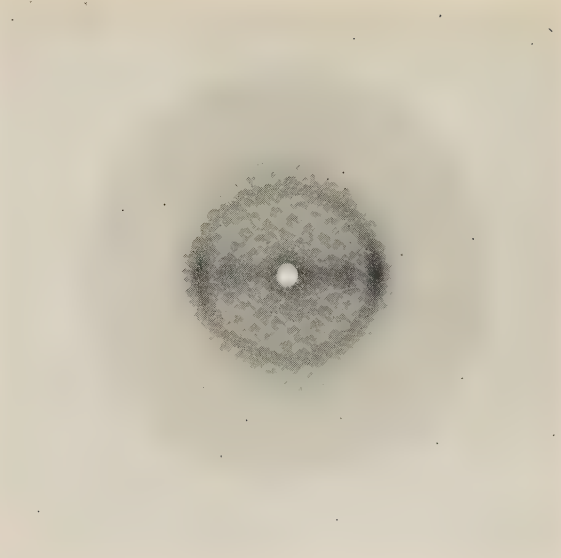
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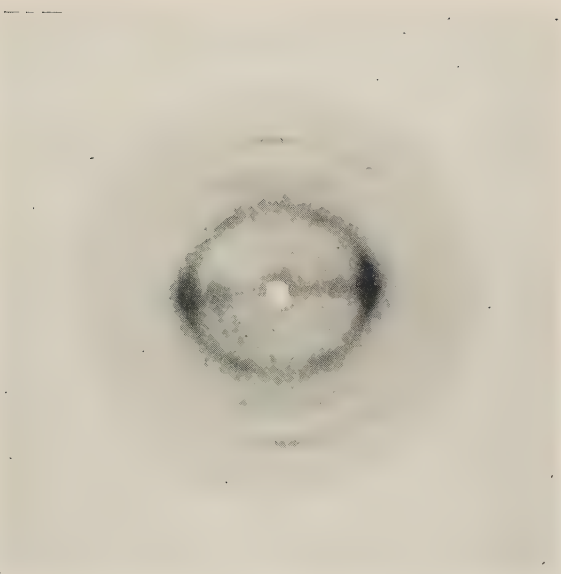
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STUDIES IN THE METABOLISM OF PLANT CELLS

VII. THE QUANTITATIVE RELATION BETWEEN SALT ACCUMULATION AND SALT RESPIRATION

By R. N. ROBERTSON* and MARJORIE J. WILKINS*

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Summary

This paper presents evidence of the quantitative relation between salt accumulation and salt respiration in plant cells.

Results of experiments on carrot tissue are given and similar results obtained by other workers with other tissues are discussed. Experiments with chlorides show that the rates of salt accumulation and of salt respiration are dependent on external concentration. When neither rate is limited by concentration, the number of molecules of salt accumulated is of the same order of magnitude as the number of electrons eliminated in salt respiration. This is based on the assumption that each oxygen molecule taken up in respiration requires four hydrogen ions and four electrons supplied by the respiratory carrier to form water.

These observations are consistent with Lundegardh's hypothesis that the electron carrier of respiration behaves as an anion carrier in accumulation, while the cations exchange with hydrogen ions. This hypothesis and the difficulties of testing it experimentally are discussed in detail; it is concluded that this hypothesis accords with most observations.

I. INTRODUCTION

The mechanism of the accumulation of salts in plant cells has been the subject of much investigation. Lundegardh (1945, 1946, 1947), in restating his hypothesis of salt accumulation, shows how the salt or anion respiration, by causing transport of anions, could provide a mechanism of accumulation. It has been shown (Robertson and Wilkins 1947) that the quantitative evidence available is not inconsistent with this hypothesis. In this paper the hypothesis will be discussed and the evidence will be given in detail.

The Lundegardh hypothesis rests on several observations:

- (1) The total respiration of tissue is greater in salt solutions than in water. This increase in respiration rate, referred to as salt respiration, has been shown in wheat roots (Lundegardh 1940), potato tissue (Steward 1937), artichoke tissue (Steward and Berry 1934), carrot tissue (Robertson 1941), and barley roots (Hoagland and Broyer 1942).
- (2) In dilute solutions, as salt respiration rate increases, the rate of salt accumulation increases.

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- (3) Inhibitor evidence suggests that the cytochrome-cytochrome oxidase system is concerned in the salt respiration and in the mechanism of accumulation (Lundegardh 1940; Hoagland and Broyer 1942; Machlis 1944; Robertson and Turner 1945).

Lundegardh suggests:

- (1) That electrons will be transported towards the surface of the cell by cytochrome in its reduced form, i.e. with ferrous iron.
- (2) That the cytochrome, after loss of an electron by oxidation, has an excess positive charge and is then available to transport an anion with unit negative charge into the cell.
- (3) That when this cytochrome is reduced with another electron, the anion will be left in the cell with the hydrogen liberated when the electron goes to the cytochrome.
- (4) That cations will be available because they will enter the cell in exchange for hydrogen ions. Hydrogen ions are finally combined with oxygen and electrons to give water.

According to this theory, both ions of a salt are accumulated, provided that the rate of ion transport exceeds the rate of back diffusion or leakage from the cell. In its simplest terms, the suggested mechanism operates by transport of anions by an electron carrier (probably cytochrome) in the direction opposite to electrons and by exchange of cations for hydrogen ions; both electrons and hydrogen ions enter into the formation of water in the respiratory process. It is necessary to postulate that the path along which the electrons (and anions) move is separated in space from the path of the hydrogen ions (and entering cations). This involves some reasonable assumptions about the nature of the membrane.

In assessing the validity of the Lundegardh theory, the quantitative relationship between the salt or anion respiration and the salt accumulation is extremely important. Lundegardh (1940) has examined the variability of the coefficient, $k = \frac{\text{anion respiration}}{\text{anion accumulation}}$ (both in g. mol.), and concludes that "the coefficient k does not give evidence of any stoichiometrical relationship between the amount of absorbed anions and the amount of oxidized glucose . . . It cannot be expected that k should show any approximate degree of constancy unless the same plants at the same stage of development and the same temperature, etc., are always used." While, as Krogh (1942) points out, discrepancies in the constancy of this coefficient cannot be used as arguments against the conception of active transport of anions by the mechanism suggested, the evidence for the Lundegardh theory will be much stronger if it can be demonstrated that, under appropriate conditions, a stoichiometrical relationship exists and if the departures from the relationship can be explained.

If the Lundegardh theory is valid, the maximum rate of accumulation of which the cell is capable should occur when each electron leaving via the cytochrome system is exchanged for an anion from the external solution. If the respiration is proceeding by a cytochrome system, all the molecular oxygen concerned in the process is combined to form water, and each molecule of oxygen therefore requires four electrons and four hydrogen ions (Ball 1944). With the normal relationship of sugar breakdown, oxygen utilization and carbon dioxide production, the equation for respiration must be written:



The maximum rate of salt accumulation should therefore be 4 g. mol. monovalent salt accumulated per g. mol. oxygen utilized or $\frac{\text{salt accumulation}}{\text{salt respiration}} = 4$. This is the reciprocal of the ratio used by Lundegardh.

II. MATERIALS AND METHODS

The materials and methods used in the collection of the data presented in this paper were similar to those described in earlier papers in this series (Robertson 1941; Robertson and Turner 1945).

The material was xylem parenchyma of carrot, *Daucus carota* L., obtained from various sources. Tissue for the experiments was prepared by cutting into discs and washing for long periods in aerated distilled water. Because of the effects of cutting, the experiments from which the results are taken included no tissue washed for less than 120 hours. In most experiments the period from cutting was between 120 and 350 hours.

The respiration was measured as oxygen uptake by standard Warburg technique or by carbon dioxide output in a continuous gas stream technique. In both techniques, aeration of the tissue was adequate to maintain the maximum aerobic respiration. A number of experiments have shown that the R.Q. of carrot tissue can be taken as unity, both in water and in solutions of monovalent salts.

Accumulation rate was measured by following either the conductivity changes or the changes in chloride concentration in the solutions surrounding the tissue. When respiration was measured by the Warburg technique, replicate sets of tissue for accumulation determinations were held in flasks in the thermostat and attached to the Warburg shaker to ensure aeration. When the respiration was measured by the continuous gas stream technique, the accumulation rate was followed by withdrawing samples of solution from the tissue vessels without interruption of the gas stream. In some experiments both chloride and conductivity methods were used and the accumulation rates so determined were in good agreement. When the conductivity method is used, it is important to note that the measurements represent the number of ions accumulated, as distinct from the total number of ions absorbed. The total number includes those entering by exchange for other ions. The agreement between chloride determinations and conductivity determinations indicated that little chloride, usually not exceeding 10 per cent., was being exchanged for other ions.

It has been pointed out previously (Robertson 1944; Robertson and Turner 1945) that there is a rapid uptake of ions immediately after the addition of salt. This initial uptake has been interpreted as a physical equilibration of the internal and external concentrations and is to be further investigated. After the internal concentration of salt approximates to the external concentration, accumulation proceeds against the concentration gradient, the rate decreasing slowly as the internal concentration increases (Robertson 1941).

The values for salt respiration — the difference between the steady rate in salt solution and the steady rate in water — are taken one and a half to two hours after adding the salt. This salt respiration in well-washed carrot tissue is usually, though not always, equivalent to the cyanide-sensitive respiration, the distilled water respiration being cyanide insensitive. Values for salt accumulation rate were taken over a few hours after the initial uptake period which lasts about half an hour after adding the salt. This is the maximum rate of accumulation and is steady over this period. The salt respiration rate is expressed as g. mol. O_2 or CO_2 /hr./g. fresh wt. and the salt accumulation as g. mol. salt accumulated/hr./g. fresh wt.

Most experiments were carried out at 25°C.; some experiments were done at a different temperature (21°C. was the lowest), and the results have been corrected to the value expected at 25°C. assuming a Q_{10} of 2 (Robertson 1944).

III. RESULTS

(a) *Experiments on Carrot Tissue*

The results of a number of experiments with different concentrations of potassium chloride are given in Table 1 and in Figure 1.

TABLE 1

Concentration of Salt (molar.)	No. of Observations	Mean Ratio		Method
		Accumulation Rate	Respiration Rate	
0.00063	2	0.44 \pm 0.16		Conductivity
0.00125	2	1.60 \pm 0.73		"
0.0025	1	2.09		"
0.005	6	2.26 \pm 0.41		"
0.01	19	2.88 \pm 0.84		{ 17 Conductivity
				{ 2 Chloride
0.02	16	2.78 \pm 0.62		{ 12 Conductivity
				{ 4 Chloride
0.03	4	3.00 \pm 0.23		Conductivity
0.04	6	3.17 \pm 0.79		{ 5 Conductivity
				{ 1 Chloride
0.05	8	3.39 \pm 0.70		Conductivity
0.06	4	3.38 \pm 0.37		"

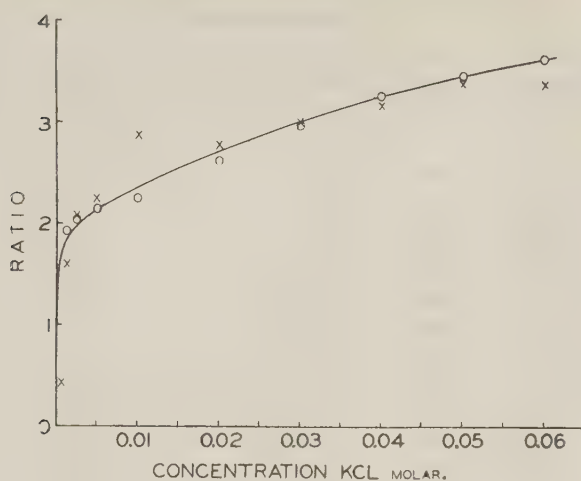


Fig. 1.—The ratio $\frac{\text{salt accumulation in g. mol.}}{\text{salt respiration in g. mol.}}$ for carrot tissue in dilute solutions of potassium chloride.

Crosses, mean ratios from Table 1; circles, ratios from lines of best fit in Figure 2.

These results were collected in the course of experiments over a long period and most of them were not specially designed to study this ratio; consequently different sets of tissue were used with the different concentrations. The results of experiments with potassium chloride, designed to test the relationship, are given in detail in Table 2.

TABLE 2

Stock of Carrots	No. of Exp.	Hours from Cutting	Concn. of Salt (molar.)	Mean Salt Respiration (g. mol./g./hr.) ($\times 10^5$)	No. of Repli- cates	Mean Salt Accumulation (g. mol./g./hr.) ($\times 10^5$)	No. of Repli- cates	Ratio
A	1	168	0.01	0.074	2	0.236	2	3.19
		168	0.03	0.109	2	0.343	2	3.15
		168	0.05	0.114	4	0.386	4	3.39
	2	216	0.01	0.079	2	0.214	2	2.71
		216	0.03	0.100	2	0.276	2	2.76
		216	0.05	0.113	4	0.388	4	3.43
	3	312	0.01	0.114	2	0.215	2	1.89
		312	0.03	0.091	2	0.284	2	3.12
		312	0.05	0.091	4	0.353	4	3.88
B	1	144	0.01	0.055	1	0.236	1	4.29
		144	0.03	0.108	1	0.349	1	3.23
		144	0.05	0.109	1	0.435	1	3.99
	2	168	0.01	0.116	1	0.244	1	2.10
		168	0.03	0.142	1	0.329	1	2.32
		168	0.05	0.108	1	0.337	1	3.12

(TABLE continued over page)

TABLE 2 (continued)

Stock of Carrots	No. of Exp.	Hours from Cutting	Concn. of Salt (molar.)	Mean Salt Respiration (g. mol./g./hr.) ($\times 10^5$)	No. of Repli- cates	Mean Salt Accumulation (g. mol./g./hr.) ($\times 10^5$)	No. of Repli- cates	Ratio
C	1	144	0.005	0.094	2	0.190	2	2.02
		144	0.01	0.117	2	0.215	2	1.84
		144	0.02	0.106	2	0.250	2	2.36
		144	0.04	0.074	2	0.300	2	4.05
		144	0.05	0.098	2	0.325	2	3.32
		144	0.06	0.100	2	0.335	2	3.35
	2	192	0.005	0.083	2	0.190	2	2.29
		192	0.01	0.128	2	0.270	2	2.11
		192	0.02	0.125	2	0.350	2	2.80
		192	0.04	0.139	2	0.365	2	2.63
		192	0.05	0.106	2	0.350	2	3.30
		192	0.06	0.120	2	0.360	2	3.00
	3	312	0.005	0.090	2	0.220	2	2.44
		312	0.01	0.114	2	0.320	2	2.81
		312	0.02	0.114	2	0.430	2	3.77
		312	0.04	0.144	2	0.430	2	2.99
		312	0.05	0.153	2	0.410	2	2.68
		312	0.06	0.128	2	0.435	2	3.40

These results were obtained with dilute solutions; since the ratio was increasing with increasing concentration, an experiment with tissue in higher concentrations was carried out on another stock of tissue. Results are given in Table 3.

TABLE 3

No. of Exp.	Hours from Cutting	Concn. of Salt (molar.)	Salt Res- piration (g. mol./g./hr.) ($\times 10^5$)	No. of Repli- cates	Salt Accumula- tion (g. mol./g./hr.) ($\times 10^5$)	No. of Repli- cates	Ratio
1	120	0.01	0.086	2	0.180	2	2.09
		0.04	0.110	2	0.290	1	2.64
		0.07	0.106	2	0.280	2	2.64
		0.10	0.108	2	0.280	2	2.59
		0.13	0.106	2	0.235	2	2.22
		0.16	0.125	2	0.280	1	2.24
2	168	0.01	0.090	2	0.220	2	2.44
		0.04	0.106	2	0.390	2	3.68
		0.07	0.110	2	0.345	2	3.14
		0.10	0.147	2	0.335	2	2.28
		0.13	0.104	2	0.340	2	3.27
		0.16	0.106	1	0.330	2	3.11

The salt respiration and salt accumulation rates are plotted against concentration in Figures 2 and 3, and the lines of best fit (by inspection) are drawn. The ratios from the smooth curves in Figures 2 and 3 are plotted in Figure 4 (Curves B and C).

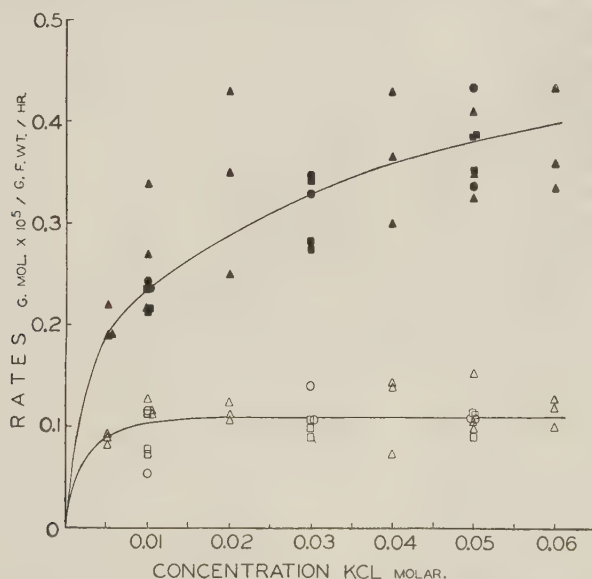


Fig. 2.—Rates of salt respiration and of salt accumulation for carrot tissue in dilute solutions of potassium chloride.

Data from Table 2: open symbols, salt respiration; solid symbols, salt accumulation; squares, stock of carrots A; circles, stock of carrots B; triangles, stock of carrots C.

A limited number of experiments with other salts, using the conductivity technique show that ratios for dilute solutions of other halides are of the same order of magnitude as those for potassium chloride. Results for an experiment in which dilute solutions of potassium chloride and calcium chloride were used on replicate sets of tissue are given in Table 4.

TABLE 4

Concentration of Salt (molar.)	No. of Observations	Salt Respiration (g. mol./g./hr.) (x10 ⁵)		Chloride Accumulation (g. mol./g./hr.) (x10 ⁵)	
		KCl	CaCl ₂	KCl	CaCl ₂
0.00063	2	0.059	0.069	0.023	0.054
0.00125	2	0.062	0.080	0.091	0.102
0.0025	1	0.061	0.079	0.128	0.256
0.005	1	0.070	0.074	0.209	0.268
0.01	4	0.108	0.116	0.319	0.240
0.02	1	0.057	0.068	0.336	0.298

The ratios calculated from the curves of best fit to these respiration and accumulation data are given in Table 5.

TABLE 5

Concentration of Salt (normal.)	Ratio	
	KCl	CaCl ₂
0.00063	0.7	0.7
0.00125	1.2	1.0
0.0025	1.9	1.6
0.005	2.8	2.6
0.01	4.0	3.4
0.02	4.2	3.4
0.04	—	3.4

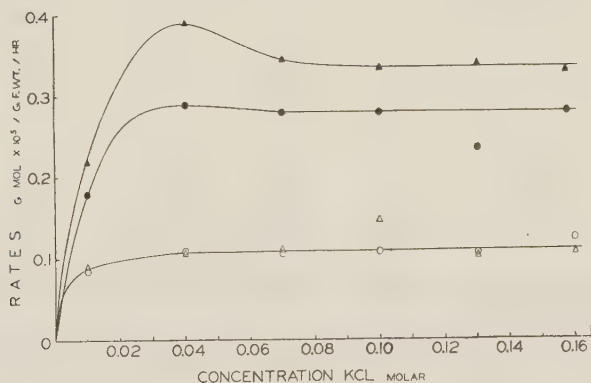


Fig. 3.—Rates of salt respiration and of salt accumulation for carrot tissue in concentrated solutions of potassium chloride.

Data from Table 3: open symbols, salt respiration; solid symbols, salt accumulation; circles, 120 hours from cutting; triangles, 168 hours from cutting.

More concentrated solutions were used in another experiment, and the results are given in Table 6.

TABLE 6

Concentration of Salt (normal.)	Salt Respiration g. mol./g./hr. ($\times 10^5$)		Chloride Accumulation g. mol./g./hr. ($\times 10^5$)		Ratio*	
	KCl	CaCl ₂	KCl	CaCl ₂	KCl	CaCl ₂
0.01	0.090	0.083	0.240	0.180	2.67	1.60
0.04	0.098	0.110	0.360	0.140	3.30	1.57
0.07	0.118	0.138	0.320	0.170	3.34	1.51
0.10	—	0.122	0.390	0.230	3.36	1.48
0.13	0.114	0.126	0.390	0.180	3.36	1.48
0.16	0.114	0.131	0.380	0.200	3.36	1.48

*The ratios given in this table are calculated from the curves of best fit to the salt respiration and accumulation data.

In other experiments, the effects of 0.01M potassium chloride, lithium chloride, and sodium chloride were compared in replicate sets of tissue. Results are given in Table 7.

TABLE 7

No. of Exp.	Salt Respiration (g. mol./g./hr.) ($\times 10^5$)			Salt Accumulation (g. mol./g./hr.) ($\times 10^5$)			Ratios		
	KCl	NaCl	LiCl	KCl	NaCl	LiCl	KCl	NaCl	LiCl
1	0.065	0.155	0.109	0.270	0.299	0.175	4.15	1.93	1.79
2	0.106	0.100	0.057	0.203	0.255	0.136	1.91	2.55	2.39
3	0.121	0.095	0.060	0.196	0.169	0.116	1.62	1.78	1.93
4	0.057	0.080	0.054	0.336	0.310	0.264	5.90	3.88	4.89
5	0.042	0.071	0.054	0.175	0.198	0.128	4.17	2.79	2.37

In some experiments, potassium chloride, potassium bromide, and potassium iodide were applied to replicate sets of tissue. The results are given in Table 8.

TABLE 8

Concentration of Salt (molar.)	No. of Observations	Ratio		
		KCl	KBr	KI
0.01	3	2.25 ± 0.45	1.86 ± 0.11	1.15 ± 0.11
	2			
	3			

The differences between potassium chloride and the other salts is not significant but the difference between potassium bromide and potassium iodide is significant at the 2 per cent. level.

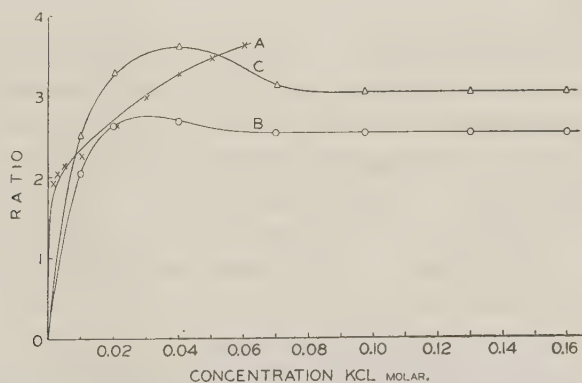


Fig. 4.—The ratio $\frac{\text{salt accumulation in g. mol.}}{\text{salt respiration in g. mol.}}$ for carrot tissue in concentrated solutions of potassium chloride.

Ratios from lines of best fit in Figure 3; circles, 120 hours from cutting; triangles, 168 hours from cutting; crosses, ratios from lines of best fit in Figure 2 for comparison.

(b) Experiments on Other Tissues

Some data were collected on beet tissue (Robertson, Turner, and Wilkins, unpublished data) and on barley roots (Milthorpe and Robertson, unpublished data). The tissue was not supplied with a range of salt concentrations but the results are worth recording here because the ratios are of the same order of magnitude as for comparable concentrations with carrot. In all experiments the salt respiration was taken as the difference between the total respiration and the respiration in water. The ratios are given in Table 9.

TABLE 9

Tissue	Salt	Concentration (molar.)	No. of Observations	Mean Ratio
Beet	KCl	0.01	5	2.63
		0.02	11	3.46
Barley Roots	KCl	0.01	10	1.29

(c) Experiments of Other Workers

Few data in the literature on salt accumulation, apart from those given by Lundegardh, are suitable for assessing the salt accumulation/salt respiration ratio. The ratio cannot be obtained from published experimental data for two principal reasons: (i) the data are insufficient to allow calculation of the difference between total respiration rate in salt and the respiration in water; and (ii) there is a lack of information about the initial or maximum accumulation rate in those instances where the rate changes markedly with time as internal concentration increases.

From the results summarized in Lundegardh's 1940 and 1945 papers, and collected from papers of Lundegardh and Burstrom, the salt respiration can be estimated in several different ways, and since all the experiments were over short periods (up to six hours), the salt accumulation is probably fairly near to its maximum. In estimating the salt respiration several different methods were used: (i) in some results the difference between the rate in salt solution and the rate in water can be obtained; (ii) in others the rate of salt respiration at different concentrations is given and an extrapolation to give the rate in zero salt concentration is possible; this assumes that the relationship of respiration to concentration is linear over the range of concentration used; and (iii) Lundegardh and Burstrom showed that the anion respiration is cyanide-sensitive, and in some results the difference between the total respiration rate in salt and the respiration in cyanide can be taken. Results obtained from Lundegardh's work are given in Table 10.

TABLE 10

Reference	Tissue	Method of Obtaining Salt Respiration Rate	No. of Observa- tions	Salt	Accumulation
					Respiration
Lundegardh (1940, p. 314)	Wheat	(ii)	3	0.002M KCl	0.31
			1	0.004M KCl	0.34
Lundegardh (1945, p. 34)	"	(ii)	3	0.002M KCl	0.34
Lundegardh (1945, p. 34)	"	(i)	3	0.001M KCl	0.70
Lundegardh (1945, p. 35)	"	(i)	1	0.001M KCl	0.24
		(i)	1	0.002M KCl	0.32
Lundegardh (1945, p. 34)	"	(iii)	2	0.002M KCl	0.46
Lundegardh (1940, p. 348)	Barley	(ii)	1	0.001M KCl	0.47
			1	0.004M KCl	1.60

Other salts investigated by Lundegardh, though giving different ratios, gave results of the same order of magnitude.

Some other workers have published results suitable for the calculation of the ratios; these are given in Table 11.

TABLE 11

Reference	Tissue	Method of Obtaining Salt Respiration Rate	No. of Observa- tions	Salt	Accumulation
					Respiration
Steward (1933, p. 208)	Potato	(i)	1	0.000468M KBr	1.4
			1	0.00468M KBr	2.0
			1	0.0468M KBr	3.2
Steward and Berry (1934)	Artichoke	(i)	1	0.00075M KBr	2.4
Van Eijk (1939)	Aster	(i)	3	0.171M NaCl	2.3
			1	0.342M NaCl	2.4

IV. DISCUSSION

(a) *Restatement of the Lundegardh Theory*

For purposes of discussion it is convenient to make a modified statement of the Lundegardh theory and this is best done by reference to a diagram (Fig. 5).

Without introducing any complicating assumptions regarding membranes, the cyclic system, fed by electrons, which is responsible for the anion transport, may be considered. When the hydrogen atom liberated by the dehydrogenase

stage of respiration reaches the cytochrome system, the electron is picked up by the cytochrome and the hydrogen ion is freed. The electron reduces the ferri-cytochrome (represented by (Fe^{+++}) , and the resulting ferro-cytochrome (represented by (Fe^{++})) may be pictured as moving towards the oxidase. At the oxidase, the electron is combined with oxygen, a hydrogen ion is picked up from the environment and attached to oxygen, and the cytochrome is oxidized to the ferri-form (Fe^{+++}) . Theorell (1947) has shown that over the range pH 3.5 to 8, the difference between ferro- and ferri-cytochrome c remains constant at one equivalent per mole. Hence the oxidized cytochrome can be pictured as carrying one

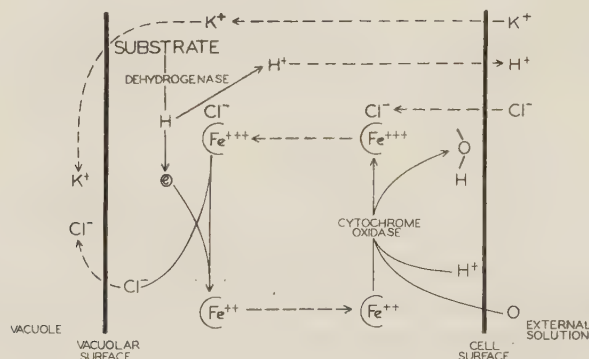


Fig. 5.—Schematic representation of the electron and anion transport system.

Solid lines represent chemical reactions; broken lines represent movements of substances; (Fe^{+++}) , oxidized cytochrome; (Fe^{++}) , reduced cytochrome.

positive charge more than the reduced form. The work of Theorell further indicates that this charge is associated through resonance with the imidazole nitrogen in the histidine of the cytochrome c protein. The oxidized cytochrome with excess positive charge will be capable of transporting an anion into the cell when it moves back to the reduction centre. This concept of movement between the oxidation and the reduction centres is introduced as an aid to the understanding of the hypothesis; the actual path may be very short.* When the cytochrome is reduced, the anion carried to the inside of the cell is left unpaired and will attract a cation. This cation could be a hydrogen ion, but since hydrogen ions readily exchange with cations from the external solution, the chances of it being a cation are high. The metallic cations can be pictured as entering by a series of exchanges along the hydrogen ion gradient; the net result would be the accumulation of both cation and anion towards the centre of the cell. This system can operate to accumulate ions only if it is assumed that the hydrogen ion path and the reduced cytochrome path are separated in the cell.

*Lundegardh (1945) has suggested that there may be an "electron wave;" this is alternative to the hypothetical movement of the redox substance, and could occur if the structural components of the cell contained electron conducting systems as suggested by Szent-Gyorgyi (1941).

Lundegardh (1940) has presented evidence for positive and negative areas in the cell surface, separated by regions of neutral molecules. It seems possible that the electron transport and the hydrogen ion paths may be associated with the extension into the cell of these different regions.

Provided that the electrons are fed to the cyclic system, anions will be conveyed through a spherical shell near the cell surface and accumulation will result. The measured rate of accumulation of anions will be the number conveyed by this system minus the number leaking from the cell by diffusion (Krogh 1946). If the leakage rate is low, at maximum accumulation the number of anions entering should approximately equal the number of electrons carried in the opposite direction. As pointed out in the introduction, the number of electrons passing through such a system will be four for each molecule of oxygen absorbed; the maximum rate of accumulation should be one molecule of monovalent salt accumulated for each electron eliminated in respiration, i.e. a ratio

$$\frac{\text{g. mol. salt accumulated}}{\text{g. mol. oxygen absorbed}} \text{ of } 4.$$

(b) *Quantitative Relation between Salt Respiration and Salt Accumulation*

(i) *The Significance of the Ratio $\frac{\text{Accumulation}}{\text{Respiration}}$* .—The data from carrot tissue show that the salt accumulation (g. mol.) and the salt respiration (g. mol. oxygen absorbed) are of the same order of magnitude and that the ratio $\frac{\text{salt accumulation}}{\text{salt respiration}}$ ranges from low values for low concentrations to nearly four for high concentrations. Similar ratios are obtained in experiments with all the salts used; in the data available from other materials, ratios of the same order of magnitude are to be seen. The ratios of salt accumulated to oxygen utilized in salt respiration are close to the hypothetical at higher concentrations. Hence the results are not inconsistent with the Lundegardh hypothesis.

(ii) *Lower Ratios at Lower Salt Concentrations*.—Since it is only at the higher concentrations of salt that the ratios approach the theoretical, the cause of lower ratios at lower salt concentrations requires examination. The lower ratios are consequences of the relations of accumulation rate and salt respiration rate to salt concentration, as illustrated in Figure 2 which is based on the data in Table 2. The curve for salt respiration approximates to the asymptote at a much lower concentration than does the curve for salt accumulation. Thus, in these experiments, salt respiration appeared to be almost at its maximum rate in a concentration of 0.02M while salt accumulation rate was still increasing with concentration. This means that the ratio for accumulation/respiration is less at lower concentrations. This observation is consistent in material from all sources and may be the explanation of Lundegardh's failure to obtain a stoichiometrical relationship between accumulation and respiration, since all his work appears

to have been done with comparatively dilute solutions. Lundegårdh himself observed this effect of concentration (cf. Lundegårdh 1940, p. 349) but placed on it a different interpretation from that given here.

(iii) *Variability in the Ratio.*—The high standard deviations (see Table 1) show the considerable variability which has been observed in ratios in different material at any one concentration of salt. The ratios will be affected by any variation or error in the estimate of either salt respiration or salt accumulation.

Estimates of salt respiration represent the difference between the total respiration in salt solution and the total respiration in water, which are both subject to errors of determination. Further, these estimates have been made on well-washed carrot tissue, in which the salt respiration is usually equal to the cyanide-sensitive respiration. In tissue which has been washed for shorter periods (up to 100 hours), a cyanide sensitive respiration, which appears soon after cutting, is still present. More investigation is required to determine how this cyanide-sensitive respiration is related to salt respiration; when this cyanide-sensitive respiration is at its maximum soon after cutting, addition of salt certainly gives no further increase. The significance of this, for present purposes, is that some persistence of this cyanide-sensitive respiration due to cutting would influence the estimate of salt respiration obtained experimentally, and Hanly and Turner (personal communication) have shown recently that the time for which this cyanide-sensitive respiration persists varies with the time of year at which the carrots are dug.

Variation in salt accumulation rate, which will also affect the ratio, may be due to the largely unknown factor of "leakage" or back diffusion from cells to solution. How far this is important, and how far it varies in different batches of carrot and in different salt concentrations, has yet to be investigated. Whatever the cause, it is certain that different sets of tissue can vary considerably in their accumulation rate at a particular concentration of salt; this is illustrated clearly in Figures 2 and 3. There is evidence that the rate of salt accumulation may be affected by time from cutting, because in Experiment C (Table 2) both salt respiration and salt accumulation increase with time from cutting over the range 144 to 312 hours. In Table 3 and in Figure 3, an increase in accumulation rate is shown between 120 and 168 hours from cutting; salt respiration did not change. Season of digging, age when dug, and time of storage of the carrots, may also have an influence. An important difference between material from different sources is illustrated in Figure 4. The Curve A, based on Table 1, shows that the ratio was still increasing at the highest concentration used, but Curves B and C, based on Table 3, show no increase in ratio at higher concentrations.

(iv) *Attainment of Hypothetical Value.*—Some factors may prevent the theoretical values for the ratio being observed. (a) *Exchange effects.* Increasing concentration externally would bring about increased ionic exchange (or re-

adjustment to new Donnan equilibria) with the cell contents; this would tend to allow entry of ions without the intervention of the accumulatory mechanism, and might therefore give false values for amount accumulated. Actually this may not be very important, particularly where the method of measuring the amount accumulated is by conductivity, since the ions entering by exchange are not recorded. If they were recorded, they would tend to make the $\frac{\text{accumulation}}{\text{respiration}}$

ratio high. (b) *Osmotic effects.* High concentrations of salt may cause the movement of water from the cells by osmosis. The amount of water withdrawn from the cell immediately after the salt is applied, and the effect of this withdrawal of water on the salt respiration and accumulation, has not yet been investigated.

(c) *Permeability effects.* The nature and concentration of the salt applied externally are likely to have important effects on the permeability of the cell membrane to ions. They may effect both the entry of ions by the accumulatory mechanism (particularly as the cation must enter by a series of exchanges) and also the leakage or back diffusion from the cell. Leakage may be increased by increasing concentrations of monovalent ions externally because of the well-known increase in permeability resulting from monovalent cations replacing divalent cations, especially calcium, in the membrane. This leakage may therefore be enhanced and operate against the hypothetical ratio being obtained at higher concentrations (see later section).

(v) *Effects of Different Salts.*—The effect of divalent cations may be summarized by saying that they stimulate respiration and chloride accumulation but, some hours after their application, the rate of accumulation falls more than can be explained by increasing internal concentration (Robertson 1941). Comparison of dilute solutions of calcium chloride with potassium chloride is given in Tables 4 and 5. Here the ratios are similar for both salts but the ratio in more concentrated solutions is less in calcium chloride than in potassium chloride (see Table 6); the effect of the longer exposure may be seen in earlier work (Robertson 1941). Lundegardh's results for experiments extending over some hours show the ratios $\frac{\text{accumulation}}{\text{respiration}}$ in the following order

$\text{Na} > \text{K} > \text{Mg} > \text{Ca} > \text{Sr} > \text{Ba}$, i.e. with the monovalents greater than the divalents. It seems possible that the lower ratios with higher concentrations or with prolonged exposure in the divalent salts are due to the divalent cations acting on the membrane, decreasing permeability, preventing the entry of further cations, and so slowing the rate of accumulation. Unequal absorption of anions and cations, with excess absorption of anions from divalent salts, has been known for some time. Ulrich (1942) has shown that excess anions are absorbed from solutions such as calcium bromide, and the cations which remain in excess in the external solution are balanced by an increase in bicarbonate ions. The failure of calcium ions to enter the cell could be due to the decreased permeability preventing exchange with hydrogen ions while the bromide is still

being accumulated. Accumulation of anions alone will not continue indefinitely, however; as Ulrich (1942) and Burstrom (1945) have shown, changes in the organic acid content occur within the cell, the acid content falling relative to the number of cations. This change in organic acid content may be related to the fall in respiration which is brought back to ground level and may even fall below it after the tissue has been in contact with the divalent cations for some hours (Robertson 1941).

Experiments on lithium chloride and sodium chloride in concentrations of 0.01M illustrate how closely salt accumulation rate is related to salt respiration rate. Though there is considerable variability between experiments, in any one experiment the respiration and accumulation by tissue in lithium chloride was always less than that in sodium chloride, averaging about two-thirds. However, the ratio of accumulation to salt respiration was the same in each. This indicates that at least in the range where salt respiration is limited by salt concentration, the accumulation rate is controlled by the salt respiration rate.

This discussion has been confined to halides because other ions are more likely to present complicated pictures. From Lundegardh's results, nitrate seems to be comparable to chloride, though there may be secondary effects with nitrate due to its participation in the cell metabolism. The differences in ratios obtained with nitrate, chloride, and sulphate may possibly be explained by differences in combining power of these ions with the cation (ferri-cytochrome) in the transport system. The observation that divalent anions such as sulphate are absorbed very slowly would be consistent with the difficulty of accumulating a divalent anion by a carrier geared to carry only one negative ion. Further, bicarbonate ions applied to the external solution would not be accumulated because it is unlikely that bicarbonate ions would exist in the acid environment of the cell in which the transport system is operating, unless there is a region of high alkalinity, which seems improbable.

(c) The Respiratory Carrier: the Nature of the Salt Respiration

While cytochrome and cytochrome oxidase are suggested as the substances responsible for the ion transport system, another electron carrier with similar characteristics would be just as suitable. The cytochrome system is suggested because (i) the accumulatory mechanism is stopped so readily by inhibitors of cytochrome oxidase, viz. cyanide, carbon monoxide, and azide (Lundegardh 1940; Machlis 1944; Robertson and Turner 1945); (ii) cytochrome represents the stage in the hydrogen transport system where the hydrogen ion and the electron become separated; and (iii) no other electron carrier has been shown to occur in carrot tissue.

The presence of a cytochrome oxidase in carrot tissue has been confirmed by isolation (Goddard, personal communication; Robertson and Bitmead, unpublished data). Attempts to isolate cytochrome c have been unsuccessful (Hanly and Turner, unpublished data). This does not necessarily imply absence

of cytochrome c, since its concentration may be quite low but still sufficient to keep pace with the comparatively low oxygen uptakes observed in plant tissue. This aspect of the work requires much more investigation. The general biological interest of the system in which a cyanide-sensitive respiration can be initiated by the addition of ions to the cell surface, has been the subject of previous comment; attention has been drawn to the similarity of this stimulated respiration to other types of stimulated respiration (Robertson and Turner 1945). It has been shown that the salt respiration is stimulated by the presence of salt in the external solution, is little affected by the salt content of the cell after accumulation, and takes a considerable time to disappear after removal of salt from the external solution. Further investigation of this stimulated respiration is suggested by the work of Goldinger and Barron (1946) in their investigation of the respiration of the fertilized sea-urchin egg. The stimulated respiration which occurs after fertilization is mediated by a cytochrome system. Goldinger and Barron suggested that the iron porphyrins are attached to structures containing highly polymerized nucleo-proteins and that the process of membrane formation is accompanied by an increase in permeability which will bring about a change in the electrolyte concentration, with subsequent depolymerization of the nucleo-proteins and liberation of cytochromes. The possibility of a liberation of cytochromes after addition of salt to carrot tissue should be investigated.

The stimulation of salt respiration to a maximum occurs at concentrations of salt which are limiting to accumulation. This suggests that the stimulated respiration is due, not to the amount of salt being transported, but to some effect of salt on the respiratory system. This is borne out by the observation (Robertson and Thorn 1945) that the salt respiration persists for some time after accumulation has ceased due to removal of salt from the external solution. Further, it was shown (Robertson 1944) that a low concentration of salt (0.01M) would give a stimulated respiration with a high Q_{10} value, suggesting that the stimulated respiration was not limited by the entry of salt.

This observation introduces one difficulty in the Lundegardh hypothesis. If the hypothesis is correct, the fact that maximum salt respiration can be reached at concentrations below those necessary for maximum accumulation, would imply that some ion, other than one from the external solution, is available for the movement of the electron carrier in the oxidized condition (Fe^{+++}). This is not an insuperable difficulty since the production of some hydroxyl or other negative ion probably occurs at the oxidase, and negative ions may be transferred towards the centre of the cell with the ferri-cytochrome, finally combining with hydrogen ions. Production of such an ion would be analogous to the production of hydroxyl ions occurs in the "alkaline wave" of the gastric mucosa (see Davies 1946). The high concentration of an ion in the external solution necessary to bring the accumulatory mechanism to full rate could be pictured as being necessitated by the competition, for the positive centres on the carrier, between naturally produced negative ions and external anions.

(d) *The Steady State Condition of Accumulation*

The mechanism which has been discussed so far is that which transports the ions from the cell surface to the interior of the cell. The net accumulation rate which is measured will be the difference between the inward rate of ion transport by such a mechanism and the rate of loss or leakage of ions in the back diffusion from the interior of the cell towards the external solution. It seems probable that the rate of leakage will vary in different cells and in the same cell at different times. In any experimental work, the results represent the average behaviour of a large number of cells, and it is possible that some cells in the material, while operating to transport ions inwards may leak more rapidly than others. Further, since increase in permeability is a common accompaniment of death, it seems probable that ageing of tissue may be accompanied by an increase in the number of "leaky" cells.

Considerable evidence suggests that the permeability to ions of plant cell membranes is very low in healthy cells. This is shown particularly in the work on the electrical resistance of the surface of *Nitella* cells (Cole and Curtis 1938). Further, as Krogh has shown with radioactive ions, the resistance of the membrane to ionic movement in *Nitella* is very high. Indirect evidence of the magnitude of the leakage from cells of carrot tissue comes from the following observation: after accumulation of salt ceases, i.e. when the external solution is replaced with water, little solute escapes to the external solution although after about 70 hours the salt respiration drops to a low value, between 25 and 30 per cent. of the maximum value in 0.01M potassium chloride (Robertson and Thorn 1945). If it is assumed that the residual salt respiration just balances the leakage of ions, i.e. if it is assumed that each g. mol. of oxygen taken up is equivalent to about four anions which would have leaked and are being replaced, then the leakage would be of the right order of magnitude to account for discrepancies between the hypothetical and observed ratios. Lundegardh has suggested that leakage from wheat roots might account for the salt respiration "idling" in water. This is based on the observation that the respiration obtained by extrapolation to zero salt concentration is often lower than the actual respiration observed in water. On this picture the salt respiration "idling" is brought about by the leakage of ions from the cell and these ions are re-accumulated by this low salt respiration. Evidence for a possible similar effect is given by beet tissue (Robertson, Turner, and Wilkins 1947) in which the respiration in water was rarely fully cyanide insensitive, and by barley roots in which the respiration in water exceeds the cyanide stable respiration by about 60 per cent. of the latter. The possibility that leakage is greater in these tissues than in carrot and is associated with a residual or "idling" salt respiration could be investigated by prolonging the cyanide inhibition of accumulation to measure the leakage. Some leakage in cyanide has been observed in barley. Loss by back diffusion from the cells will contribute to departures from the hypothetical relationship and should be con-

sidered in any attempt to explain the form of the accumulation/time curves, particularly as the rate of leakage will increase as the internal concentration increases and the external concentration decreases.

(e) *The Lundegardh Hypothesis and Alternative Hypotheses*

The Lundegardh hypothesis has features in common with several earlier hypotheses. One suggestion (Brooks 1929, 1937) is that of an exchange of cations for hydrogen ions and of anions for bicarbonate ions. There are, however, a number of objections to this theory. Briggs (1930) pointed out that the suggested mechanism would not operate in a membrane with a mosaic of positive and negative areas but only in a membrane alternatively positive and negative in time. Even if this were assumed, there are other objections; because of the hydrogen ion characteristics of the cell surface, it is doubtful if there is much bicarbonate ion leaving the cell and it is likely that most of the carbon dioxide leaves as the molecule in solution. Even if exchange with the bicarbonate ion were possible, increased production of carbon dioxide alone is not capable of increasing accumulation. This is shown by experiments with methylene blue where carbon dioxide output is increased without any accompanying increase in accumulation rate (Hoagland and Broyer 1942; Briggs and Robertson, unpublished data). This may be very significant since the methylene blue may replace or by-pass the cytochrome system.

The total energy necessary to bring about accumulation at the observed rates is a very small fraction of that liberated in the salt respiration. Robertson (1941) has shown that the calculated energy required to effect accumulation from 0.01M potassium chloride was only about one per cent. of that freed. This low energy utilization is consistent with the view that the accumulatory mechanism is an incidental consequence of a particular respiratory mechanism. The system of a carrier as suggested by Lundegardh seems to be satisfactory to apply the energy to accumulation. It is similar in principle, though not in detail, to the one suggested by Wohl and James (1942). Lundegardh's hypothesis meets a number of the requirements imposed by other observations. For instance, it has been postulated that both the relation of accumulation rate to concentration and the changing rate of accumulation with internal concentration in time can be explained on the basis of a constant concentration layer at or near the surface of the cell (Robertson 1941). This layer would correspond to the saturated anion pick-up centres. The hypothesis also explains the observation by Lundegardh (1940) that the accumulatory mechanism can be inhibited by the application of electric current; electric current in the appropriate direction would prevent electrons from being moved to the surface and, therefore, as observed, would depress the salt respiration rate.

The hypothesis can also be applied to certain analogous processes in animal tissues. The possible applications were reviewed by Krogh (1946).

V. CONCLUSION

This paper presents evidence of the quantitative relation between salt accumulation and salt respiration; it is shown that the number of molecules of salt accumulated is of the same order of magnitude as the number of electrons eliminated in salt respiration; this in itself does not constitute proof of Lundegardh's hypothesis that the electron carrier of respiration behaves as an anion carrier, but is consistent with it. Since the hypothesis is in accord with other data it must be concluded that it is the most promising yet suggested. More work is necessary to show conclusively (a) that an electron carrier operates as the anion carrier and (b) that, if there is such a carrier, it is part of the cytochrome system.

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VII. REFERENCES

- BALL, E. G. (1944).—*Ann. N.Y. Acad. Sci.* 45: 363.
BRIGGS, G. E. (1930).—*Proc. Roy. Soc. B.* 107: 248.
BROOKS, S. C. (1929).—*Protoplasma* 8: 389.
——— (1937).—*Trans. Faraday Soc.* 33: 1,002.
BURSTROM, H. (1945).—*Ark. Bot.* 32A (7): 1.
COLE, K. S., and CURTIS, S. J. (1938).—*J. Gen. Physiol.* 22: 37.
DAVIES, R. C. (1946).—*Biochem. J.* 40: xxxv.
ELJK, M. VAN (1939).—*Rec. Trav. Bot. Neerland.* 36: 561.
GOLDINGER, J. M., and BARRON, E. S. G. (1946).—*J. Gen. Physiol.* 30: 73.
HOAGLAND, D. R., and BROYER, T. C. (1942).—*Ibid.* 25: 865.
KROCH, A. (1946).—*Proc. Roy. Soc. B.* 133: 140.
LUNDEGARDH, H. (1940).—*Ann. Agric. Coll. Sweden* 8: 234.
——— (1945).—*Ark. Bot.* 32A (12): 1.
——— (1946).—*Nature* 157: 575.
——— (1947).—*Ann. Rev. Biochem.* 16: 503.

- MACHLIS, L. (1944).—*Amer. J. Bot.* 31 (3): 183.
- MILTHORPE, J., and ROBERTSON, R. N. (1948).—*Aust. J. Exp. Biol. Med. Sci.* (in press).
- ROBERTSON, R. N. (1941).—*Ibid.* 19: 265.
- (1944).—*Ibid.* 22: 237.
- and TURNER, J. S. (1945).—*Ibid.* 23: 63.
- and THORN, M. (1945).—*Ibid.* 23: 305.
- , TURNER, J. S., and WILKINS, M. J. (1947).—*Ibid.* 25: 1.
- and WILKINS, M. J. (1948).—*Nature* 161: 101.
- STEWART, F. C. (1933).—*Protoplasma* 18: 208.
- (1937).—*Trans. Faraday Soc.* 33: 1,006.
- and BERRY, W. E. (1934).—*J. Exp. Biol.* 11 (2): 103. ,
- SZENT-GYORGYI, A. (1941).—*Nature* 148: 157.
- THEORELL, H. (1947).—*Advances Enzym.* 7: 265.
- ULRICH, A. (1942).—*Amer. J. Bot.* 29: 220.
- WOHL, K., and JAMES, W. O. (1942).—*New Phytol.* 41: 230.

STUDIES ON THE NITROGEN METABOLISM OF PLANTS

VII. TOXICITY OF SOME OXIMES AND OXIMINO-ACIDS TO *AZOTOBACTER* AND THEIR UTILIZATION

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Summary

Twenty-two different oximino-derivatives were synthesized; they included ketoximes, dioximes, oximino-monocarboxylic acids, and oximino-dicarboxylic acids and esters of the last two groups of compounds. Toxicity of these compounds to *Azotobacter* was measured; esters, dioximes, and α -oximino-dicarboxylic acids were non-toxic at 5×10^{-3} M concentration, whilst the other oximino-derivatives were toxic at about 5×10^{-4} M. Toxicity was related to chemical structure and probable orientation in relation to membrane surfaces.

It is shown that *Azotobacter* readily uses both *cis*- and *trans*- modifications of both oximinosuccinic and α -oximinoglutaric acids as source of nitrogen at pH 6.8 in absence of atmospheric nitrogen or other source of nitrogen.

The possibility that these two oximino-acids might be intermediates in nitrate metabolism is discussed.

I. INTRODUCTION

Nitrogen added to plants either as ammonium or nitrate ion results in increases in amounts of both proteins and amino-acids and usually also in the amides asparagine and glutamine. However, evidence is accumulating which suggests that differences in metabolism occur according as the plants are provided with either ammonium salts or nitrates.

One of the most striking of these differences is in the relative amounts of organic acids, especially malic and citric acids, contained in the tissues. These acids decrease in amount as the concentration of ammonium salt in the external solution is increased (Clark 1936; Vickery *et al.* 1940).

In leaves of plants supplied with ammonium salts, Wood and Petrie (1938) showed that at a steady state the relationship between amino-acid and ammonium content within the leaves could be expressed by a logarithmic function of ammonia-N. The asymptotic part of the curve was ascribed to the fact that substrates providing the carbon-skeleton were limiting. It has long been held that oxy- and keto-acids are the immediate non-nitrogenous precursors of amino-acids. Wood, Mattner, and Symons in 1947 (unpublished data) supplied varying treatments of both ammonium salt and nitrate to oat plants under controlled environmental conditions; at the steady state they found that the same relationship between proteins and amino-acids held for both "ammonium" and "nitrate" plants. The ammonium plants showed the same relations between amino-acids

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and ammonia contents as described by Wood and Petrie (loc. cit.) ; but in nitrate plants the amino-acid content was independent of the ammonium content (which remained small) but increased as the nitrate supply was increased. In both sets of plants amide content showed similar relationship to ammonia-N content.

Pearsall and Billimoria (1939) asserted that light caused increased nitrate reduction as measured by increased production of organic-N in detached leaves floating on nutrient solutions.

Blackman and Templeman (1940) recorded gain of protein in leaves in full sunlight when plants were supplied with either ammonium salts or nitrate, but at lower light intensities they found that elaboration of protein was reduced and nitrate accumulated. They concluded that shaded leaves of grasses lose their ability to elaborate nitrate into organic-N, but their ability so to elaborate ammonium is unimpaired. Their ability to reduce nitrate was not dependent on carbohydrate content.

Burstrom (1943), using wheat seedlings at the two-leaf stage, found that nitrate in leaves was not reduced in darkness, but in light in amounts proportional to the light intensity. In this respect, he points out that leaves differ from roots, for in the latter, nitrate reduction occurs in darkness. Burstrom measured the rate of photosynthesis and also the amounts of soluble sugars in the leaves; he found, over a given time interval, that the amount of photosynthesis was greater than the increase in sugar content and that the difference between them was proportional to the amount of nitrate reduced. The amount of nitrate reduced was independent of rate of respiration; in the absence of nitrate he claims that all the carbon dioxide is converted into sugars. When ammonium salts were present these were assimilated by the leaf in both light and darkness at the expense of sugars. He concluded that nitrate was not reduced to ammonia since stored sugars were not drawn on in nitrate nutrition, and suggested that a direct carbon-nitrogen assimilate was formed in leaves in the presence of light, and also suggested, though without any experimental evidence, that a reduction product—nitrous acid, hyponitrous acid, or hydroxylamine—might combine with a photosynthetic product to form an oxime.

All the experiments quoted above provide at least some grounds for concluding that alternative mechanisms may exist for the formation of organic-N, and especially of amino-acids, according as the plant is supplied with ammonium salts or nitrates. Accordingly we decided to investigate the utilization of some oximes by plants, and especially of oximino-acids which might be considered to be intermediaries in amino-acid synthesis. For preliminary investigations on toxicity and utilization we used *Azotobacter*, since use of a bacterium gave results in a short time and also because it has been claimed that oximes are produced as intermediaries in the metabolism of this organism.

Investigators concerned with the mechanism of N-fixation or nitrate reduction in N-fixing bacteria fall into two groups: those who consider ammonia to be the primary product of reduction, and those who assign this role to hydroxylamine. Arguments advocating ammonia as the primary product are necessarily

inconclusive since ammonia is always produced within the organism by deamination of amino-acids. The chief proponent of the hydroxylamine theory is Virtanen (1939). A critical review of the position has been made by Burris and Wilson (1945) who suggest theoretical pathways for N-fixation which may not be mutually exclusive.

Virtanen's arguments, derived from consideration of the relative amounts of oxalacetic and α -ketoglutaric acids formed by *Rhizobium* and their relative powers of condensing with hydroxylamine to form oximes, are unconvincing. By far the strongest claim for his theory is his reported isolation of *trans*-oximinossuccinic acid from nodules of *Rh. leguminosarum*.

Endres (1935) isolated a carboxime (amounting to 10 per cent. of the total-N fixed) from medium in which *Azotobacter* was growing. He found that this compound in concentrations from 1×10^{-5} M to 1.2×10^{-4} M (the highest concentration investigated) had no effect upon the oxygen uptake by the bacteria and concluded that it originated from N-fixation rather than from cell katabolism. Burk and Horner (1936) disagreed with this explanation on the ground that *Azotobacter* will not assimilate hydroxylamine even in non-toxic concentration and that the oxime was formed not only when molecular N was used as a source of N, but also when nitrate, nitrite, or ammonia was used. They considered, therefore, that the oxime was an unspecific by-product of metabolism.

Burris (1942) has shown that *Azotobacter* in air enriched with N^{15} produces the highest level of N^{15} in glutamic acid and the next highest in aspartic acid; findings which suggest the important roles of dicarboxylic acids and which he suggests are evidence for the "ammonia" theory, although of course the evidence is not exclusive. So far as *Azotobacter* is concerned it is possible that both mechanisms are operative, viz through an oxime or through ammonia. Virtanen (1947) has recently expressed the view that it is not impossible that hydroxylamine is chiefly reduced to ammonia and that oximes are formed to a small extent through by-reactions with hydroxylamine.

II. EXPERIMENTAL METHODS

(a) *Synthesis of Oximino-Compounds*

With one or two exceptions it was necessary to synthesize and carefully purify all the oximes used by us. In some cases these syntheses, using methods adopted by other workers, proved difficult and gave poor yields, and we were compelled to try alternative procedures. Since many of these compounds have seldom been prepared we give brief accounts of our experience.

Acetoxime, 2-butanone oxime and 2-pentanone oxime were obtained from the corresponding ketones by the prolonged action of ice-cold aqueous alkaline hydroxylamine solution according to standard procedure. Methyl ethyl ketone was obtained readily by distillation of mixed calcium salts of acetic and propionic acids, but a mixture of calcium acetate and calcium butyrate gave negligible quantities of methyl propyl ketone which was obtained in quantity by alkaline

hydrolysis of ethyl α -ethylacetoacetate. Methylglyoxime was obtained readily from chloracetone and hydroxylamine according to the method of Hantzsch and Wild (1896). Merck's dimethylglyoxime was recrystallized and used as source of this compound. Diacetylmonoxime was readily obtained from methyl ethyl ketone by the method of Diels and Jost (1902).

The method of Meyer and Jenny (1882) for α -oximinopropionic acid using aqueous solutions of sodium pyruvate and hydroxylamine gave very poor yields but the oximino-acid was obtained in 90 per cent. yield by treating an alcoholic solution of hydroxylamine hydrochloride with sodium ethylate and adding the filtered solution to an equivalent amount of pyruvic acid dissolved in absolute alcohol, both solutions being cooled to 0°C. On standing in a refrigerator overnight the acid was obtained as silky crystals.

α -Bromobutyric acid warmed with hydroxylamine hydrochloride in alkaline solution according to the method of Hantzsch and Wild (1896) gave negligible quantities of α -oximinobutyric acid. The acid was obtained in fair yield by hydrolysis of the ester obtained by passing nitrous gases through an alcoholic solution of ethyl α -ethylacetoacetate, a modification of Furth's (1883) method for α -oximinovaleric acid. The latter acid was prepared readily by Furth's (loc. cit.) method from ethyl nitrite and ethyl α -propylacetoacetate.

γ -Oximinovaleric acid was obtained in 60 per cent. yield according to the method of Muller (1883). However, the reaction is much slower than there suggested, and better yields were obtained by standing the reaction mixture overnight in a refrigerator.

$\gamma\delta$ -dioximinovaleric acid was obtained in good yield from dibromolaevalinic acid according to the method of Wolff (1890).

Ethyl α -oximinopropionate was obtained by refluxing the silver salt of the acid with ethyl iodide. The ethyl esters of α -oximinobutyric and α -oximinovaleric were obtained in the course of preparation of the corresponding acids. The ethyl ester of α -oximinoacetoacetic acid was obtained in good yield from acetoacetic ester and NaNO_2 according to the method of Wolff (1902).

Trans-oximinosuccinic acid.* The direct action of alkaline hydroxylamine on oxalacetic (oxyfumaric) acid gave an uncrystallizable oil which rapidly became deep red. The acid was obtained in good yield by forming the Piutti ester by adding concentrated hydroxylamine solution to the sodium derivative of ethyl oxalacetate and finally completing the saponification according to the method of Cramer (1891). This oximino-acid is unstable in acid solution, rapidly decomposing into water and CO_2 , and depositing deep red crystals of cyanoacetic acid; however, we found it quite stable in neutral or alkaline solutions.

Cis-oximinosuccinic acid† was obtained in about 50 per cent. yield from dinitrososuccinylsuccinic diethylester after saponification with sodium ethylate

*This acid is recorded by Beilstein as β -oximinosuccinic acid.

†This acid is recorded by Beilstein as α -oximinosuccinic acid.

and decomposition of the silver salt according to the method of Ebert (1885). The ethyl ester of this acid was obtained in the course of preparation of the acid.

α -Oximinoglutaric acids. Our experience generally had been that the direct addition of hydroxylamine to α -keto-acids or their esters gave rise to uncrystallizable oils so that the effect of direct addition to hydroxylamine to α -ketoglutaric acid was not immediately tried. Small quantities of an α -oximinoglutaric acid were synthesized by condensing ethyl β -iodopropionate with acetoacetic ester and then condensing the resulting diethyl α -acetoglutarate with ethyl nitrite to form the oxime ester (Wislicenus and Grutzner 1909). This method was abandoned owing to the small yields (about 15 per cent.) of α -acetylglutaric ester obtained. The oximino-acid was also prepared from dibromolaevulinic acid (the latter according to Heil and Kehrner (1884); the method of Wolff (1885) is too slow and unsatisfactory), through $\gamma\delta$ -dioximinovaleric acid, furazanpropionic acid and δ -cyano- γ -oximinobutyric acid according to the methods of Wolff (1890). The lowest yield in this sequence was about 50 per cent., but owing to the long sequences of reactions involved, the amount of oximino-acid obtained was small. The acids obtained by these methods both melted at 156°C.

Finally, satisfactory yields (about 75 per cent.) of an α -oximinoglutaric acid were obtained by direct addition of a cold concentrated solution of hydroxylamine hydrochloride to a cold concentrated solution of α -ketoglutaric acid. We have found best conditions for synthesis of the latter to be synthesis of triethyl oxalosuccinate according to the method of Wislicenus (1911) (the method of Blaise and Gault (1911) gives very poor yields) followed by its decomposition by the method of Blaise and Gault (1911). The oximino-acid crystallizes readily in white rosettes. This acid melts at 140°C., whereas that formed by the first two methods melts at 156°C. We believe the latter to be the *trans*- form and the acid melting at 140°C. to be the *cis*- form.

Several attempts were made under a variety of conditions to obtain β -oximinoglutaric acid from acetonedicarboxylic acid and hydroxylamine according to the method of Emery (1890), but in all cases an uncrystallizable oil was obtained.

In all preparations, and especially where hydroxylamine was used in preparation, it was necessary to purify the compounds carefully before testing for toxicity to *Azotobacter*. Purification was ensured by a final recrystallization from ether.

(b) Cultural Technique

The organism used was *Azotobacter chroococcum*, obtained from the Waite Agricultural Research Institute and labelled Strain C. Active cultures were maintained by sub-culturing once a week and inoculations of experimental media were made from one-day-old cultures.

The medium was prepared as follows: 0.8 g. K_2HPO_4 , 0.2 g. KH_2PO_4 , 0.2 g. $MgSO_4 \cdot 7H_2O$, and 0.1 g. $CaSO_4 \cdot H_2O$ were dissolved in a litre of water and the mixture allowed to settle. To 210 ml. of the supernatant solution 0.125 g. lactic acid neutralized with NaOH, 2.0 g. sucrose, and a trace of $FeCl_3$ and $Na_2MoO_4 \cdot 2H_2O$ were added and the solution made up to 250 ml. Lots of this solution, each of 20 ml., were placed in Erlenmeyer flasks and autoclaved. Where oximino-acids were to be added to the medium these were first dissolved in water, neutralized with NaOH solution, sterilized in a Seitz filter, and the appropriate amount added to the medium in the flasks. The final reaction of the medium was pH 6.8. The medium was inoculated with 2 drops (approximately 0.08 ml.) of a one-day-old culture from a Pasteur pipette and cultured at 24°C. for 2-3 days.

For estimation of toxicity at different concentrations of oximes and oximino-acids, the sole criterion was whether or not growth occurred—visible growth being readily detected by turbidity in the medium. All treatments were replicated.

In determining utilization of oximino-acids by *Azotobacter*, culture flasks were prepared as above containing different concentrations of the substances under examination. After inoculation these were placed in a large desiccator which was evacuated, refilled and washed with cylinder oxygen (which contains too much nitrogen to use as the experimental atmosphere), the desiccator again evacuated and partially filled with pure oxygen obtained by heating pure MnO_2 and $KClO_3$. The pressure of oxygen in the desiccator was approximately 0.5 atm.

The amount of nitrogen utilized under these conditions was determined by precipitating the organisms with 10 per cent. $Al_2(SO_4)_3$ followed by successive centrifugations and washings according to the procedure of Horner and Allison (1944); the precipitate was then digested and the nitrogen estimated by micro-Kjeldahl.

III. RESULTS AND DISCUSSION

(a) *Toxicity of Oximino-Derivatives to Azotobacter*

Results of the toxicity of these compounds, as measured by failure of *Azotobacter* to grow, are set out in Table 1.

It is clear that so far as toxicity is concerned, the compounds examined fall into four groups:

- (1) Hydroxylamine, toxic at a concentration of $1 \times 10^{-5}M$.
- (2) Ketoximes, toxic at concentrations of about $10^{-3}M$.
- (3) α -Oximino-monocarboxylic acids, toxic at about $10^{-4}M$.
- (4) Substances non-toxic at $5 \times 10^{-3}M$ and in some cases non-toxic at $10^{-2}M$. These compounds include esters of α -oximino-mono- and di-carboxylic acids, α -oximino-dicarboxylic acids, diacetyl monoxime, and the dioximes methylglyoxime, dimethylglyoxime and $\gamma\delta$ -dioximinovaleric acid.

TABLE 1

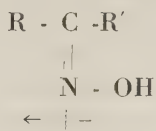
TOXICITY OF OXIMINO-DERIVATIVES TO AZOTOBACTER

Oximino-Derivatives	Molarity									
	1×10^{-2}	5×10^{-3}	2×10^{-3}	1×10^{-3}	5×10^{-4}	2×10^{-4}	1×10^{-4}	5×10^{-5}	1×10^{-5}	5×10^{-6}
Hydroxylamine		—		—				—	—	+
<i>Ketoximes</i>										
Acetoxime		—		—		+	+	+	+	+
2-propanone oxime		—		—		+	+	+	+	+
2-butanone oxime			—	—	±	+	+	+	+	+
Diacetyl monoxime	+		+	+	+	+	+	+	+	+
Methylglyoxime	—		+	+	+	+	+	+	+	+
Dimethylglyoxime	(not soluble)		+	+	+	+	+	+	+	+
<i>Oximino-monocarboxylic acids</i>										
Oximinopropionic				—		—	—	+	+	+
α -Oximinobutyric				—		±	+	+	+	+
α -Oximinovaleic	—			—		—	±	+	+	+
γ -Oximinovaleic				—		+	+	+	+	+
$\gamma\delta$ -Dioximinovaleic										
<i>Esters</i>										
Ethyl oximinopropionate	±	+		+			+	+	+	+
Ethyl α -oximinobutyrate		+		+			+	+	+	+
Ethyl α -oximinovaleate	+		+	+			+	+	+	+
Ethyl oximinoacetoacetate	±	+		+			+	+	+	+
Ethyl oximinosuccinate	—	+		+			+	+		
<i>Oximino-dicarboxylic acids</i>										
Cis-oximinosuccinic	—	±		+			+	+	+	+
Trans-oximinosuccinic	±	+		+			+	+	+	+
Cis- α -oximinoglutaric		±		+			+	+	+	+
Trans- α -oximinoglutaric	—	+	+	+			+	+	+	+
+ growth occurred — no growth ± growth incipient										

Before suggesting possible mechanisms of inhibition of growth it is necessary to show that penetration of the cells by the oximes occurs. Taking mortality as an indication of penetration it is clear that the ketoximes are approximately equally effective in spite of a marked increase in lipophilic properties in the series, 2-butanone oxime being only sparingly soluble in water. A similar argument, so far as approximately equal toxicity is concerned, holds for the α -oximino-monocarboxylic acids, but here supporting evidence of penetration is obtained from the fact, to be discussed later, that oximinopropionic acid and also the oximino-dicarboxylic acids in non-toxic concentration can be utilized as a source of nitrogen by plants.

It should be pointed out here that α -oximino acids are relatively strong acids, comparable to monochloroacetic acid, for example. The following are the

dissociation constants ($K \times 10^5$) of some of the acids used in this investigation: α -oximinopropionic, 51.4; α -oximinobutyric, 69; α -oximinovaleric, 68.5; *trans*-oximinosuccinic, 110; *cis*-oximinosuccinic, 372. Dissociation constants for the corresponding unsubstituted acids are propionic, 1.43; butyric, 1.49; *n*-valeric, 1.61; succinic (K_1), 6.8 γ -Oximinovaleric acid on the other hand is a weak acid ($K \times 10^5 = 2.3$). It is clear, therefore, that the oximino group as a substituent is definitely acylous, i.e. electron-attracting; it is analogous in this respect to the amino group which, though basic, is definitely acylous as a substituent, as might be expected in view of the fact that the nuclear charge on the nitrogen atom is greater than that on the carbon. It is also apparent that as usual the dissociation constant is determined by the distance of the substituent from the carboxyl group. In oximes both trivalent N and OH are dipoles and dipoles are therefore directed as follows:



The oximino group is not ionized though the oximino-acids are strongly ionized.

According to modern views, the living cell surface consists of a lipid-protein film with the lipid as layers of molecules with their hydrophilic groups directed towards the water and their lipophilic chains directed inwards and interlocking; the proteins are spread at the surface of the lipoids. It also seems probable that there is a definite spatial arrangement of enzymes in the cell and that some of these are formed by proteins on either side of the lipid layer, e.g., succinic dehydrogenase.

If this view is correct, interpretation of the inhibition of growth brought about in *Azotobacter* by oximino compounds may be explained by orientation and combination or coordination of the polar molecules or ions of the oximes with specific centres of the enzymes or proteins, although these may be modified by the structure of the oximes themselves. It is possible, for example, that the oxime group may coordinate with semi-polar $> \overset{+}{\text{C}} - \overset{-}{\text{O}}$ groups in the specific protein.

Hydroxylamine is in a different category from the other oximes studied here; first, it is a kationic substance, and second, it may form oximes by condensation with keto groups. Endres (1935) found that oxygen uptake by *Azotobacter* was depressed by about 60 per cent. by 10^{-5}M hydroxylamine, figures which agree with our growth studies. It is also of interest to note here that Nakamura (1938) found 95 per cent. inhibition of photosynthesis in *Chlorella* by 10^{-4}M hydroxylamine, and Gaffron (1942) has indicated that it affects an oxygen-liberating enzyme and that its effect is not due to oxime formation but to specific interaction with the photochemical enzyme.

We suggest that the $=\text{NOH}$ group of the ketoximes coordinates with groups on the specific protein, and in any case these molecules would be oriented with the hydrophilic $=\text{NOH}$ toward the protein and their lipophilic hydrocarbon chains directed inward.

Similar considerations apply to the α -oximino-monocarboxylic acids, which, however, are strongly ionized and contain in addition to an $=\text{NOH}$ group the hydrophilic $-\text{COO}^-$ group with a tendency to coordinate with, for example, NH_2 groups on the protein. The hydrocarbon chains, however, will be directed towards the lipoids, as in the case of ketoximes.

In α -oximino-dicarboxylic acids, however, the situation is different. These compounds are strong acids, highly ionized, and with two $-\text{COO}^-$ groups, one at each end of the molecule which is consequently less polar. The ion will therefore tend to coordinate with kationic groups on the specific protein and to orient itself parallel to the surface of the protein, a state of affairs unlike that of the oximino-monocarboxylic acids with their ions approximately at right angles to the protein surface. In the dicarboxylic acids, whether an $=\text{NOH}$ group can bond or not with an appropriate surface group will depend upon the topography of the active surface. Such an explanation offers a reasonable basis for the relative toxicities of the oximino derivatives of the mono- and di-carboxylic acids.

However, this theory does not explain the non-toxicity of other substances examined, viz. esters of oximino-acids, dioximes, and diacetylmonoxime. It is true that in most of these compounds (except the esters) the presence of hydrophilic groups at each end of the molecule will cause it to lie on the protein surface, but does not explain why coordination of the oxime group with the protein may not occur.

We suggest that the relative non-toxicity of these compounds is due to their structure in which chelation occurs. Chelation—the inter- or intra-molecular coordination in systems containing donor and acceptor units—has been shown by infra-red spectroscopy to occur in dioximes (eg. in dimethylglyoxime and benzil dioxime) and in keto-oximes (e.g. benzil monoxime). Since chelation is similar to that leading to simpler open-chain coordination complexes, this process will compete with coordination of oximino groups with any active centres of a specific protein and accounts for the non-toxicity to *Azotobacter* of methyl- and dimethylglyoxime, $\gamma\delta$ -dioximinovaleric acid and diacetyl monoxime. We suggest that the lack of toxicity of the esters of oximino-acids is also due to chelation since their structure is akin to that of a keto-oxime like diacetyl monoxime, for the introduction of the ethyl group into the carboxyl destroys the resonance of the latter. Pertinent structures are shown in Figure 1.

(b) Utilization of Oximino-Acids as Source of Nitrogen

The highest non-toxic concentration of hydroxylamine and oximino-propionic acid at which growth of *Azotobacter* occurred was respectively $5 \times 10^{-6}\text{M}$ and $5 \times 10^{-5}\text{M}$; these correspond respectively to 0.0014 mg. N and

0.014 mg. N per 20 ml. culture solution. To determine whether utilization of these compounds occurred in complete absence of other sources of nitrogen is beyond the limits of accuracy of the methods employed. In a subsequent paper it will be shown that these two compounds are utilized by oat plants in non-toxic concentrations.

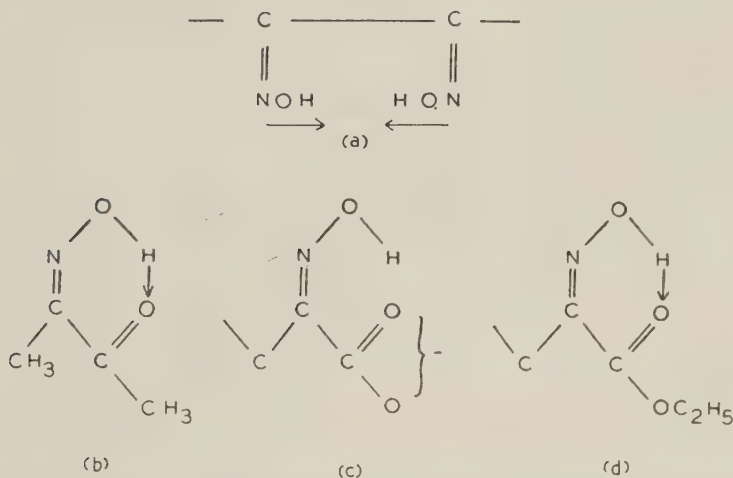


Fig. 1.—Illustrating the structural formulae of (a) dioximes; (b) diacetylmonoxime; (c) ion of an α-oximino-acid; (d) ester of an α-oximino-acid.

In *Azotobacter* cultures, the question whether the organism can utilize oximino-acids when deprived of all other forms of nitrogen was restricted to the oximinosuccinic and oximinoglutaric acids, where, owing to their relative non-toxicity, much larger concentrations can be employed. The organisms were grown, under the conditions described earlier, under a partial pressure of pure oxygen, in media containing the oximino-acids in $5 \times 10^{-3}M$, $1 \times 10^{-3}M$ and $5 \times 10^{-4}M$ concentration; these concentrations correspond to 1.4 mg., 0.28 mg., and 0.14 mg. of nitrogen per 20 ml. of culture solution. The N-contents of organisms grown at different concentrations at different incubation times are given in Table 2. The figures represent the amount of N derived from the oximino-acid and were obtained by deducting the N-content of the controls (which contained no added N in the medium) from that of the organisms grown on the oximes; the value of the controls in each experiment was approximately 0.05 mg. N.

Table 2 shows that the oximino-acids are utilized as a source of nitrogen and that the amount of organic nitrogen elaborated by the organism tends to increase both with concentration of the oximino-acid and with time. The experiments also indicate that *trans*-oximinoglutaric acid is more readily utilized than *trans*-oximinosuccinic acid and both of these more readily than *cis*-oximinoglutaric acid.

TABLE 2

AMOUNTS OF ORGANIC NITROGEN (MG. N) ELABORATED BY AZOTOBACTER GROWN ON 20 ML. CULTURE SOLUTION OXIMINO-ACIDS AT VARIOUS CONCENTRATIONS FOR DIFFERENT TIMES; pH 6.8.

Culture Solution	Age (hr.)	Molarity of Oximino-acid				
		In Oxygen			In Air	
		5×10^{-3}	1×10^{-3}	5×10^{-4}	5×10^{-3} Nil (Control)	
<i>Trans</i> -oximinosuccinic acid	48	0.03	0.02	nil	0.18	0.17
	72	n.d.*	0.03	0.02	n.d.	n.d.
	72	0.03	0.05	n.d.	0.17	0.14
	84	0.13	0.09	n.d.	1.53	1.43
	120	0.13	0.09	0.11	0.38	0.47
	120	0.50	0.09	0.09	1.01	0.46
<i>Trans</i> -oximinoglutaric acid	48	0.09	0.02	nil	0.24	0.17
	72	0.37	0.06	0.02	0.78	0.26
	72	0.12	0.05	0.02	0.58	0.14
	84	0.41	0.16	0.01	1.80	1.43
<i>Cis</i> -oximinoglutaric acid	120	0.03	0.09	0.05	0.06	0.10
	120	nil	0.09	0.12	0.50	0.47

*n.d.—not determined.

The fifth column in Table 2 shows the amount of N fixed in air by cultures, run at the same time as those in the absence of atmospheric N, but with the addition of 5×10^{-3} M oximino-acid. These figures show that, at 5×10^{-3} M concentration N-synthesis from the oximino-acids is considerably less in oxygen than in air.

The sixth column in Table 2 gives N-content of organisms grown in air with no added N. In all cases differences between columns 5 and 6 are small compared with the total amount of N fixed, but generally are positive, which may mean that a small amount of N from the oximino-acids is utilized even when the organism is fixing N in air.

IV. REFERENCES

- BLACKMAN, G. E., and TEMPLEMAN, W. G. (1940).—*Ann. Bot.* 4: 533.
 BLAISE, E. E., and GAULT H. (1911).—*Bull. Soc. Chim.* 9: 451.
 BURK, D., and HORNER, C. K. (1936).—*Soil Sci.* 41: 81.
 BURRIS, R. H. (1942).—*J. Biol. Chem.* 143: 509.
 ——— and WILSON, P. N. (1945).—*Ann. Rev. Biochem.* 14: 685.
 BURSTROM, H. (1943).—*Ann. Agric. Coll. Sweden* 11: 1.
 CLARK, H. E. (1936).—*Plant Physiol.* 11: 5.
 CRAMER, C. (1891).—*Ber. dtsh. chem. Ges.* 24: 1,198.
 DIELS, O., and JOST, H. (1902).—*Ibid.* 35: 3,292.
 EBERT, H. (1885).—*Liebigs Ann.* 229: 65, 76.
 EMERY, W. O. (1890).—*Ber. dtsh. chem. Ges.* 23: 3,765.

- ENDRES, D. (1935).—*Liebigs Ann.* 518: 109.
- FURTH, A. (1883).—*Ber. dtsh. chem. Ges.* 16: 2,180.
- GAFFRON, H. (1942).—*J. Gen. Physiol.* 26: 195.
- HANTZSCH, A., and WILD, W. (1896).—*Liebigs Ann.* 289: 293.
- HEIL, C., and KEHRER, E. A. (1884).—*Ber. dtsh. chem. Ges.* 17: 1,981.
- HORNER, C. K., and ALLISON, F. E. (1944).—*J. Bact.* 47: 1.
- MEYER, V., and JENNY, A. (1882).—*Ber dtsh. chem. Ges.* 15: 1,527.
- MULLER, A. (1883).—*Ibid.* 16: 1,618.
- NAKAMURA, H. (1938).—*Acta Phytochim., Tokyo* 10: 271.
- PEARSALL, W. H., and BILLIMORIA, M. C. (1939).—*Ann. Bot.* 3: 601.
- VICKERY, H. B., PUCHER, G. W., WAKEMAN, A. J., and LEAVENWORTH, C. S. (1940).—*Conn. Agric. Exp. Sta. Bull. No.* 442.
- VIRTANEN, A. I. (1939).—*Trans. 3rd Comm. Int. Soc. Soil Sci., A*, p. 4.
- (1947).—*Biol. Rev.* 22: 239.
- WISLICENUS, W. (1911).—*Ber. dtsh. chem. Ges.* 44: 1,564.
- and GRUTZNER, R. (1909).—*Ibid.* 42: 1,939.
- WOLFF, L. (1885).—*Liebigs Ann.* 229: 249.
- (1890).—*Ibid.* 260: 79.
- (1902).—*Ibid.* 325: 134.
- WOOD, J. G., and PETRIE, A. H. K. (1938).—*Ann. Bot.* 2: 729.

THE COPPER-CATALYSED OXIDATION OF ASCORBIC ACID IN FRUIT AND VEGETABLE SUSPENSIONS

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Summary

The copper-catalysed oxidation of ascorbic acid was studied in phthalate and phosphate buffers. The rate increased with concentration of copper up to ten parts per million and with increasing pH up to 6.0.

Oxalic, malic, citric, and tannic acids, sulphur dioxide, albumen, cystein, and thiourea reduced copper catalysis. Outstanding "protection" was given by thiourea.

Most fruit and vegetable tissues contain substances which reduce copper catalysis. Onion tissue gives outstanding "protection" against low concentrations of copper.

I. INTRODUCTION

Considerable attention has been given to the retention of ascorbic acid by fruit and vegetables, both fresh and processed. The stability of ascorbic acid to oxidation can vary greatly in different tissues and under different conditions of processing and storage. The factors concerned in this stability include (a) access of atmospheric oxygen, as determined by the structure of the tissue and by processing procedures; (b) oxidation catalysts, including enzymes, copper, and other substances in the tissues; and (c) "protective" factors.

Krishnamurthy and Giri (1941a) reported the existence of both oxidizing and "protective" factors in various vegetables. They separated the oxidizing enzymes from the "protective" factor by precipitation with acetone. They found that the "protective" factor was thermostable and inhibited the copper-catalysed but not the enzyme-catalysed reaction.

This paper is concerned with the copper-catalysed oxidation of ascorbic acid and its modification by various "protective" substances. Barron *et al.* (1936a) found that ascorbic acid is not autoxidizable in acid or neutral solutions up to pH 7. They tested the catalytic effect of salts of manganese, nickel, iron, cobalt, calcium, and copper at pH 4-6 and found that copper alone has a marked catalytic effect. Barron *et al.* (1936b) found that some animal and vegetable fluids contain protective agents, including glutathione, proteins, and amino-acids, which inhibit copper catalysis. Krishnamurthy and Giri (1941b) found oxalic acid, xanthine, uric acid, theophylline, creatinine, antipyrine, and albumen to have

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powerful "protective" action. Kawereau and Fearon (1944) investigated various "protective" agents and found the most effective to be thiourea. They separated volatile thiol compounds from cabbage, and found them to be effective protectors of ascorbic acid.

The kinetics of the copper-catalysed oxidation have been studied by Silver-Blatt, Robinson, and King (1943). They showed that if k (the velocity constant) does not exceed 0.12 min.^{-1} , the reaction rate is directly proportional to the concentrations of cupric ion and ascorbic acid, but the k values depend in a rather complex manner upon the initial concentrations of ascorbic acid, hydrogen ion, and oxygen. The k values tend to decrease with increasing initial concentrations of ascorbic acid. These authors found that appreciable concentrations of hydrogen peroxide accumulate during the course of the reaction, but this does not affect the rate if k is less than 0.12 min.^{-1} .

Weissberger and Lu Valle (1944) found that the relation between pH and rate is rather complex. The results indicated that only the monovalent ion of ascorbic acid is the substrate of copper catalysis.

Our studies are concerned first with the copper-catalysed oxidation of ascorbic acid in buffer solutions of varying pH, and then with the "protective" effect of added substances and of fruit and vegetable suspensions. For assessing "protective" effects, it is necessary to have reference solutions in which copper catalysis is at a maximum. Phthalate buffers from pH 2.2 to 6.0 and phosphate buffers of pH 6.0 and 7.0 were found fairly satisfactory for this purpose. These solutions gave a rate of oxidation which generally increased regularly with increasing pH and was considerably higher than that given by other solutions containing "protective" substances.

II. EXPERIMENTAL PROCEDURE

All media used for studying the oxidation of ascorbic acid were prepared with glass distilled water. Clark and Lubs' 0.05M phthalate buffers were prepared with a slight modification. Sulphuric acid was used in place of hydrochloric acid in buffers of pH 2.2 and 3.0. Mapson (1941) showed that chloride had a variable effect on the oxidation, increasing it in lower and decreasing it in higher concentration. By substituting sulphuric acid, oxidation in the phthalate buffers was found to decrease regularly with decreasing pH. Sorenson's M/15 phosphate buffers were also prepared.

The "protective" effect of a number of substances which occur naturally or are recommended for preservation was investigated in phosphate buffer of pH 6.0. The substances tested were starch, sucrose, glucose, oxalic acid, malic acid, citric acid, tannic acid, albumen, cystein, thiourea, and sulphur dioxide. The concentrations of the naturally occurring substances approximated to those

which might be present in fruit and vegetable suspensions. After addition of oxalic, malic, or citric acid to the buffer solution, the pH was readjusted to 6.0 with sodium hydroxide. No adjustment was required with the other substances.

The fruit and vegetable suspensions were prepared by disintegrating one part by weight of tissue with four parts of glass distilled water in the Waring Blender. They were boiled for two minutes to inactivate the enzymes. (Practically identical rates of oxidation were obtained after boiling for twenty minutes). The rose hip suspension was prepared from one part of rose hips and nine parts of water as more concentrated suspensions were too viscous to handle. Only one sample of each fruit and vegetable was investigated.

Studies of the oxidation of ascorbic acid were made as follows: Fifty millilitres of solution or suspension, containing 0, 0.5, 1, 2, 5, and 10 parts of copper per million were measured into a 100 ml. florence flask and brought to 40°C. in a constant temperature bath. The copper was added as copper sulphate. Air was bubbled at the rate of approximately 12 ml. per second. After adding 2 ml. of 0.5 per cent. ascorbic acid, a 5 ml. aliquot was pipetted into 10 ml. of 3 per cent. metaphosphoric acid and titrated with approximately 0.001N 2,6-dichlorophenolindophenol. Subsequent aliquots were pipetted at intervals up to 30 minutes and titrated with the dye.

When the logarithm of the ascorbic acid concentration c was plotted against time t , an approximately linear curve was obtained, indicating a first order reaction. The velocity constant k was calculated from the formula $1/t \log_e C_0/C_t$ and expressed as min.^{-1} .

To avoid foaming in some of the suspensions, a few drops of caprylic alcohol were added to the medium. This was found not to affect the rate appreciably.

In these studies, the initial concentration of ascorbic acid was approximately 20 mg. per 100 ml. in all preparations except the rose hip suspension. In most cases this concentration of ascorbic acid was derived almost wholly from the added ascorbic acid, as little ascorbic acid remained in the suspension after blending. The exceptions were diluted orange juice and rose hip suspension. The former retained approximately 20 mg. of ascorbic acid per 100 ml. and did not require any further addition. The latter retained approximately 80 mg. of ascorbic acid per 100 ml.

III. OXIDATION IN PURE SOLUTIONS

The velocity constants in phthalate and phosphate buffers are given in Table 1, and the effect of pH on the velocity constant with one and ten parts of copper per million is given in Figure 1.

In all buffers the velocity constant increased considerably with increasing copper concentration. Copper was present in the control buffers up to 0.2 part per million, and was probably responsible for the slight oxidation. For each level of copper the velocity constant increased with increasing pH up to pH 6. The velocity constant was still higher at pH 7 for the lower levels of copper (up to 2 p.p.m.), but it fell off at the higher levels, probably due to precipitation.

TABLE I
VELOCITY CONSTANT IN PHTHALATE AND PHOSPHATE BUFFERS

Buffer	Added Copper (p.p.m.)				100 <i>k</i> at pH				
					2.2	3.0	4.0*	5.0	6.0
Phthalate	-	-	-	0	0.0	0.1	0.4 (0.2)	1.4	1.9
				0.5	0.2	0.7	4.1 (2.6)	10.3	17.8
				1.0	0.2	1.2	5.3 (3.6)	14.4	25.1
				2.0	0.4	2.0	8.5 (5.2)	17.9	33.0
				5.0	0.8	5.0	15.4 (8.2)	27.4	43.2
				10.0	1.7	11.7	26.5 (11.3)	41.2	127.3
Phosphate	-	-	-	0				0.5	0.9
				0.5				34.6	53.0
				1.0				49.0	72.2
				2.0				77.9	102.5
				5.0				120.3	118.5
				10.0				161.2	131.3

*The velocity constants in brackets are those obtained when 100 mg. of ascorbic acid per 100 ml. are added initially.

At pH 6, copper catalysis was definitely greater in phosphate than in phthalate buffer. This indicates a specific effect of the anion in addition to the effect of the hydrogen ion. However, catalysis in these buffers still provides an approximate standard for reference, as it is considerably greater than that occurring in the presence of certain "protective" substances which reduce the concentration of free copper ions by chemical combination.

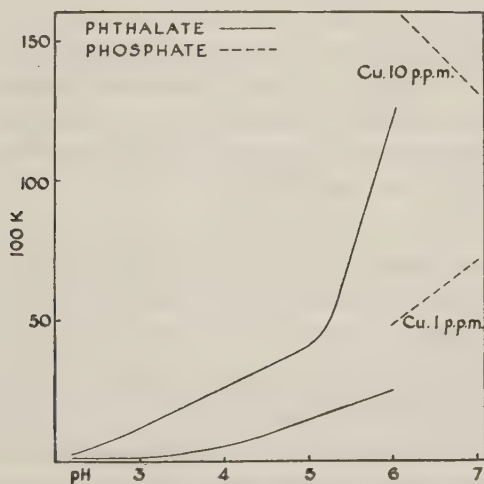


Fig. 1.—The effect of pH on the velocity constant of the oxidation of ascorbic acid by copper.

Most of the velocity constants refer to an initial concentration of 20 mg. of ascorbic acid per 100 ml. At a higher concentration (100 mg./100 ml.), the velocity constant is definitely less.

The velocity constants in phosphate buffer of pH 6.0 with various added substances are given in Table 2. The "protective" effects can be estimated by comparison with the velocity constant in the control buffer.

TABLE 2
VELOCITY CONSTANT IN PHOSPHATE BUFFERS AT pH 6.0 WITH ADDED SUBSTANCES

Added Substances	100 <i>k</i> with Added Copper Equivalent to					
	Nil	0.5 p.p.m.	1.0 p.p.m.	2.0 p.p.m.	5.0 p.p.m.	10.0 p.p.m.
Control - - -	0.5	34.6	49.0	77.9	120.3	161.2
Starch (1%) - - -	1.4	28.4	42.0	63.5	96.9	102.6
Sucrose (1%) - - -	1.6	36.5	46.1	53.0	102.5	148.0
Glucose (1%) - - -	0.7	35.8	51.7	65.6	115.1	123.6
Oxalic acid (0.1%) - - -	0.1	2.1	3.6	5.8	8.4	9.3
Malic acid (0.2%) - - -	2.8	17.1	21.6	30.6	44.0	55.3
Citric acid (0.2%) - - -	1.4	6.2	7.3	9.2	10.4	12.5
Tannic acid (0.02%) - - -	0.0	1.7	2.6	4.5	7.9	10.2
Albumen (0.2%) - - -	0.0	0.3	0.6	1.5	8.9	53.5
Cystein, HCl (0.02%) - - -	0.3	0.3	1.2	1.6	5.0	9.2
Thiourea (0.02%) - - -	0.0	0.1	0.0	0.1	0.4	0.5
Sulphur dioxide (0.02%) - - -	0.0	19.5	24.5	35.7	44.9	57.6

The "protective" effects of starch, sucrose, and glucose were found to be negligible, as the velocity constants differed only slightly from those of the control buffers. All the other substances showed some "protective" effect. Good "protection" was afforded, at the concentrations tested, by oxalic acid, citric acid, tannic acid, and cystein. Albumen was very effective in low concentrations of copper, but less effective at higher concentrations. Outstanding "protection" was afforded by thiourea, which at a concentration of 0.02 per cent. resulted in negligible oxidation with up to two parts of added copper per million and only slight oxidation with ten parts per million.

The "protective" effect of cystein is not confined to the reduced form, as the disulphide cystin was found to be almost as effective. With ten parts of copper per million, an equivalent concentration of cystin gave a value of 12.4 for 100 *k*.

IV. OXIDATION IN FRUIT AND VEGETABLE SUSPENSIONS

The velocity constants in fruit and vegetable suspensions are given in Table 3. In some cases the centrifuged liquor was used, as the whole suspension was difficult to pipette and titrate. The suspensions were centrifuged at 2,000 r.p.m. Centrifuging tends to reduce the protective effect.

TABLE 3

VELOCITY CONSTANT IN SUSPENSIONS BOILED FOR TWO MINUTES

Preparation	pH	Original Copper (p.p.m.)	Nil	100 <i>k</i> with Added Copper Equivalent to				
				0.5	1.0	2.0	5.0	10.0
				p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.
Apple suspension -	3.2	0.2	1.3	1.5	1.9	2.5	4.3	8.5
Orange juice suspension	3.6	0.1	0.0	0.6	1.5	2.8	4.7	6.6
Rose hip liquor - -	3.6	0.2	0.3	0.8	1.6	1.8	3.2	3.9
Tomato suspension -	4.3	0.2	0.7	2.2	4.6	6.3	8.8	10.5
Orange rind liquor -	5.1	0.2	0.2	1.3	2.9	6.4	12.9	18.7
Onion suspension -	5.3	0.1	0.0	0.0	0.0	0.5	4.4	7.1
Parsley liquor - -	5.5	—	0.3	0.8	1.1	2.6	12.5	22.7
French bean liquor -	5.5	0.2	0.3	0.5	0.8	2.9	7.9	11.0
Potato suspension -	5.7	0.2	0.9	0.9	0.9	2.1	4.0	8.0
Cabbage suspension -	6.0	0.2	0.4	0.8	0.9	3.1	6.7	13.0
Asparagus liquor - -	6.0	0.3	0.5	1.0	2.0	4.9	11.9	19.9
Swede turnip suspension	6.0	0.1	0.2	1.7	3.1	5.8	11.8	17.0
Silver beet liquor -	6.7	0.4	1.2	1.4	1.7	2.2	5.7	13.2

Most of the tissues contain substances which reduce the copper-catalysed oxidation of ascorbic acid (as compared with oxidation in phthalate and phosphate buffers). The rate of oxidation in onion tissue was negligible with one part of copper per million and very low with higher concentrations. Rose hips gave a particularly low rate in ten parts of added copper per million. Orange juice also gave a low rate of oxidation. Tomato gave comparatively poor protection against low concentrations of added copper.

As the rate of the copper-catalysed oxidation varies considerably with pH, the "protective" effect of a particular suspension can only be estimated by comparing its velocity constant with that of a phthalate or phosphate buffer of the same pH. The latter can be obtained from the curves in Figure 1. Using these data, the ratio k_s/k_b , where k_s is the velocity constant of the suspension and k_b is the velocity constant of a phthalate or phosphate buffer of the same pH, has been calculated for one and ten parts of copper per million. The results are given in Table 4.

From Table 4 it appears that the comparatively low rate of oxidation in orange juice is due as much to the low pH as to the effect of "protective" substances. In the rose hip suspension, the comparatively high initial concentration of ascorbic acid (80 mg./100 ml.) also reduced the velocity constant. The "protective" effect is particularly noticeable in the products of high pH.

Organic acids, tannins, and sulphur compounds probably all contribute to the "protective" effect. One would generally expect their specific effect to be less at lower pH, due to the reduced ionization and copper binding power.

TABLE 4
THE PROTECTIVE EFFECT IN FRUIT AND VEGETABLE SUSPENSIONS

Preparation	Reference Buffer	pH	k_s/k_b with Added Copper Equivalent to	
			1 p.p.m.	10 p.p.m.
Apple suspension - -	Phthalate	3.2	1.07	0.57
Orange juice suspension - -	"	3.6	0.48	0.32
Rose hip liquor - -	"	3.6	0.52	0.19
Tomato suspension - -	"	4.3	0.62	0.34
Orange rind liquor - -	"	5.1	0.19	0.43
Onion suspension - -	"	5.3	0.00	0.13
Parsley liquor - - -	"	5.5	0.06	0.30
French bean liquor - -	"	5.5	0.04	0.14
Potato suspension - -	"	5.7	0.04	0.08
Cabbage suspension - -	"	6.0	0.04	0.10
Asparagus liquor - -	"	6.0	0.08	0.16
Swede turnip suspension -	"	6.0	0.12	0.13
Silver beet liquor - -	Phosphate	6.7	0.03	0.09

There appeared to be little specific "protection" in the apple suspension. As shown previously, the "protective" effect of 0.2 per cent. malic acid is comparatively low. Moreover, there is some evidence of a thermostable catalyst besides copper in the apple suspension. The velocity constant (1.3) for the apple suspension without added copper is higher than would be expected from its copper content (0.2 p.p.m.) particularly as an additional one part of copper per million only increased it to 1.9.

The high "protection" of onion tissue is probably due largely to the volatile sulphur compounds associated with pungency. A buffered distillate of the same volume and pH as the original suspension was found to give complete "protection" against one part per million of added copper ($100k = 0.0$). Suspensions of non-volatile residue obtained by prolonged boiling or by blending oven dried tissue still showed some "protective" effect. With one part of added copper per million, $100k_s = 2.1$ and $k_s/k_b = 0.12$.

V. DISCUSSION

It is probable that the copper-catalysed oxidation of ascorbic acid takes place in two stages. Cupric ion oxidizes the ascorbic acid to dehydroascorbic acid and is reduced to cuprous. The cuprous is then oxidized to cupric by atmospheric oxygen. Silverblatt, Robinson, and King (1943) showed that if k is not greater than 0.12 min.^{-1} , the rate is proportional to the concentration of cupric ion. Most of the "protective" substances are known to form complexes which reduce the concentration of free cupric ions.

The results for fruit and vegetable suspensions indicate generally a reduced copper catalysis compared with the reference buffers. In most cases the effect of low concentrations of copper is still quite appreciable. This applies par-

ticularly to tomato products. The relation of these studies to copper contamination in fruit and vegetable products can only be approximately assessed. Oxidation can be minimized by rigorous exclusion of air, but where this is impossible, copper contamination should be reduced to a minimum.

This paper makes a further contribution to the already considerable literature on copper catalysis and the effect of "protective" substances. Various additional substances and natural products have been tested for their "protective" effect. In addition, an attempt has been made to estimate the "protective" effect more precisely by comparison with copper catalysis in certain buffer solutions of the same pH. These solutions were found to give approximately maximum catalysis.

VI. ACKNOWLEDGMENT

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VII. REFERENCES

- BARRON, E. S. G., *et al.* (1936*a*).—*J. Biol. Chem.* 112: 625.
——— (1936*b*).—*Ibid.* 116: 563.
KAWEREAU, E., and FEARON, W. R. (1944).—*Sci. Proc. R. Dublin Soc.* 23: 171.
KRISHNAMURTHY, P. V., and GIRI, K. V. (1941*a*).—*J. Ind. Chem. Soc.* 18: 7.
——— (1941*b*).—*Ibid.* 18: 191.
MAPSON, L. W. (1941).—*Biochem. J.* 35: 1,332.
——— (1945).—*Ibid.* 39: 228.
SILVERBLATT, E., ROBINSON, A. L., and KING, C. G. (1943).—*J. Amer. Chem. Soc.* 65: 137.
WEISSBERGER, A., and LU VALLE, J. E., (1944).—*Ibid.* 66: 700.

THE ENZYME-CATALYSED OXIDATION OF ASCORBIC ACID IN FRUIT AND VEGETABLE SUSPENSIONS

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Summary

Enzymic oxidation of ascorbic acid is particularly rapid in orange rind, parsley, french bean, and cabbage suspensions. It is only slight in onion and swede turnip, and negligible in orange juice, rose hip, and tomato suspensions. Apple suspensions give very variable results.

In many suspensions the oxidase activity is far greater than could be due to the copper concentration, if present in ionized form.

The ascorbic acid oxidase of cabbage has an optimum pH of approximately 6. In suspensions of cabbage in water the activity is mainly in the insoluble particles, but the solubility is increased in the presence of neutral salts. The enzyme is precipitated by saturation with ammonium sulphate.

Evidence has been obtained that the mechanism of oxidation in apple suspensions is largely direct.

I. INTRODUCTION

In a previous paper (Huelin and Stephens 1948), the authors described studies of the copper-catalysed oxidation of ascorbic acid in fruit and vegetable suspensions. In the work on copper catalysis, the suspensions were boiled for two minutes to inactivate the enzymes. The relative importance of enzymic oxidation in unboiled suspensions is discussed in the present paper.

Enzymes which oxidize ascorbic acid have been found in a number of tissues. Johnson and Zilva (1937) found that cabbage, cauliflower, cucumber, and marrow contain enzymes which oxidize ascorbic acid directly. They showed that apple and potato tissue did not oxidize ascorbic acid directly but contained phenolases which oxidized ascorbic acid in the presence of catechol or juice. However, Hackney (1946) has obtained evidence of a true ascorbic acid oxidase (which can oxidize ascorbic acid directly) in apple juice.

Highly active preparations of ascorbic acid oxidase have been obtained by various workers. Tauber, Kleiner, and Mishkind (1935) obtained the enzyme from an extract of Hubbard squash by precipitation with acetone. Highly purified preparations have been obtained by Lovett-Janison and Nelson (1940) and by Powers, Lewis, and Dawson (1941). The purified preparations were found to contain 0.15-0.24 per cent. of copper. Tadokoro and Takasugi (1939) obtained the enzyme in crystalline form.

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Szent-Gyorgyi (1931) precipitated an ascorbic acid oxidase from cabbage extract by saturation with ammonium sulphate. This enzyme oxidized ascorbic acid directly and was devoid of polyphenolase activity.

As the most highly purified preparations of ascorbic acid oxidase contain definite amounts of copper, this enzyme is regarded by some workers as a copper protein complex. Meiklejohn and Stewart (1941) found that the ascorbic acid oxidase activity of cucumber juice was proportional to the non-dialysable copper but greater than the catalytic activity of the copper alone. Other workers, however, have doubted the existence of a specific oxidase and claimed that the activity is simply due to copper either alone or in non-specific association with protein. Stotz, Harrer, and King (1937) found copper-gelatin or copper-albumen mixtures to simulate most of the properties of ascorbic acid oxidase. The catalytic activity of such mixtures was, however, less than that of the copper alone.

As the work described in this paper is primarily a study of enzymic oxidation such as would occur during fruit and vegetable processing, i.e. in the preparation of pulps and juices, and in the partial disintegration of tissue for freezing, drying, or canning, it follows that only enzymes which are active in the disintegrated tissue are considered. It is possible for enzymes to be inactive at the pH of the minced tissue or tissue extract but highly active in regions of different pH which may occur in the intact cells.

II. EXPERIMENTAL PROCEDURE

The fruit and vegetable suspensions were prepared in the Waring Blender from one part by weight of tissue and four parts of glass distilled water. However, the rose hip suspension was found too viscous to handle, and had to be diluted to half this concentration before use.

The measurements of oxidation were carried out at 40°C. as previously described (Huelin and Stephens 1948). After adding 2 ml. of 0.5 per cent. ascorbic acid to 50 ml. of suspension, the usual procedure was to pipette aliquots of the suspension into 3 per cent. metaphosphoric acid at definite intervals and titrate with 2.6 dichlorophenolindophenol. Some suspensions, however, were difficult to pipette and titrate, and a modified procedure was necessary. This consisted of adding metaphosphoric acid solution to the whole of the suspension after a definite time interval and making up to volume with metaphosphoric acid. An aliquot of the centrifuged liquor was taken for titration. To determine the ascorbic acid present at zero time, metaphosphoric acid was first added to the suspension followed by 2 ml. of 0.5 per cent. ascorbic acid and a similar procedure followed. (It is essential to study the oxidation in whole suspensions, as most of the enzyme activity may be in the suspended particles).

The results for unboiled suspensions were expressed as per cent. oxidation of ascorbic acid in five minutes. This was preferred to a velocity constant, as the reaction kinetics were not similar in all cases. The per cent. oxidation in suspensions boiled for two minutes, which is due to traces of copper or other

thermostable catalysts, was subtracted to obtain the true enzymic oxidation. It was assumed that the enzymic and non-enzymic catalysis were independent.

III. OXIDATION IN DIFFERENT TISSUE SUSPENSIONS

The enzymic oxidation of ascorbic acid in various fruit and vegetable suspensions is given in Table 1.

TABLE 1
ENZYMIC OXIDATION IN FRUIT AND VEGETABLE SUSPENSIONS

Suspension	pH of Unboiled Suspension	Copper Concentration (p.p.m.)	Per Cent. Oxidation in 5 Minutes
Apple	3.2	0.2	88.5
Orange juice	3.6	0.1	0
Rose hip	3.6	0.2	0
Tomato	4.3	0.2	0
Orange rind	4.9	0.2	59.7
Onion	5.4	0.1	1.0
Parsley	5.7	—	88.6
French bean	6.1	0.2	71.0
Potato	5.9	0.2	13.1
Cabbage	5.9	0.2	93.8
Asparagus	6.2	0.3	20.1
Swede Turnip	6.3	0.1	1.9
Silver beet	6.7	0.4	39.1

These data indicate very high enzyme activity in apple, orange rind, parsley, french bean, and cabbage suspensions; only slight activity in onion and swede turnip suspensions; and negligible activity in orange juice, rose hip, and tomato suspensions.

In the initial experiments, when single samples of a number of different fruits and vegetables were compared, the apple suspension gave a very high enzyme activity. On further investigation of a number of apple samples considerable variation was experienced. The per cent. oxidation in five minutes for thirteen apple suspensions of pH 3.2-3.7 was found to vary from 3 to 91, with a mean of 24. It is of considerable interest to find a definite oxidase activity in this range of pH, as neither ascorbic acid nor polyphenol oxidase is usually found active below pH 4.

In many of the suspensions the oxidase activity is far greater than would be expected if all the copper were in the free ionic form. This is particularly noticeable in the apple, french bean, and cabbage suspensions (Table 1), which all had a copper content of 0.2 part per million. If the enzyme is inactivated by boiling, the addition of even ten parts of copper per million fails to restore the original level of oxidation. The results are shown in Table 2.

TABLE 2
COMPARISON OF ENZYMIC AND COPPER CATALYSIS

Suspension	Enzyme	Per Cent. Oxidation in 5 Minutes	
		Copper (1 p.p.m.)	Copper (10 p.p.m.)
Apple	88.5	9.0	34.5
French bean	71.0	3.8	42.3
Cabbage	93.8	4.3	47.8

If the oxidation is due to copper enzymes, they must hold the copper in a special type of combination, which considerably enhances its catalytic effect. Considerably more than non-specific association with protein is involved as the addition of gelatin or albumen can actually reduce the catalytic effect of copper (Stotz, Harrer, and King 1937; Hueilin and Stephens 1948).

IV. THE ASCORBIC ACID OXIDASE OF CABBAGE

The per cent. oxidation in five minutes in four cabbage suspensions of pH 5.9-6.4 varied from 45 to 94. Oxidation in the centrifuged liquor was only a fraction of that in the whole suspension and varied from 0 to 10 per cent. Most of the enzyme activity appears to be in the insoluble particles. The enzyme in the original suspension was found to be completely inactivated in 30 seconds at 100°C.

The effect of pH on the activity of the insoluble enzymes was determined very readily. The suspension was centrifuged at 2,000 r.p.m. and the insoluble portion washed twice with glass distilled water. It was then made up to the same volume as the original suspension with citrate-phosphate buffer of the appropriate pH. Studies of oxidation gave the following results (Table 3).

TABLE 3
EFFECT OF pH ON CABBAGE OXIDASE ACTIVITY

pH	Per Cent. Oxidation in 5 Minutes
3	2.3
4	0.8
5	50.2
6	65.7
7	45.4
8	22.4

Maximum activity was obtained at pH 6, which was approximately the pH of the original cabbage suspension. The relation is similar to that obtained with ascorbic acid oxidase of Hubbard squash (Tauber, Kleiner, and Mishkind 1935).

In suspensions of cabbage in water, not more than one-tenth of the enzyme activity was in the soluble portion. The solubility of the enzyme was considerably increased in the presence of neutral salts. The addition of 0.5M sodium chloride,

potassium chloride, or ammonium sulphate to the suspension increased the proportion of soluble enzyme to about half. These salts reduced the pH by about 0.2. After adding the salt, the suspension was allowed to stand at 1°C. for two hours before centrifuging. The effect of various concentrations of sodium chloride is shown in Table 4.

TABLE 4
EFFECT OF SODIUM CHLORIDE ON SOLUBILITY OF CABBAGE OXIDASE

Sodium Chloride (Concentration)	Per Cent. Oxidation in 5 Minutes in Suspension (S)	Centrifuged Liquor (L)	Proportion of Soluble Enzyme (L/S)
Nil	67.9	7.2	0.11
0.1M	91.4	17.2	0.19
0.2M	94.6	30.5	0.32
0.3M	86.4	40.9	0.47
0.4M	89.9	51.7	0.57
0.5M	70.5	36.7	0.52
0.6M	70.9	28.0	0.39
0.8M	62.9	26.4	0.42
1.0M	57.4	20.8	0.36

Maximum solubility was obtained in 0.4M sodium chloride.

Sodium chloride also brings about a gradual inactivation of the enzyme, and the suspensions and liquors should be held at a low temperature except when measurements of oxidation are actually being made. Concentrations of sodium chloride up to 1.0M gave negligible loss of activity in six hours at 1°C, but considerable loss at 40°C. The loss at 40°C. is shown in Table 5.

TABLE 5
INACTIVATION OF CABBAGE OXIDASE BY SODIUM CHLORIDE AT 40°C.

Hours at 40°C.	Per Cent. Oxidation in 5 Minutes in Suspensions in		
	Water	0.4M NaCl	1.0M NaCl
0	67.1	79.0	56.3
3	61.0	40.6	32.1
6	56.4	19.5	14.5

The solution of enzyme in 0.4M sodium chloride was purified with barium acetate and the enzyme was precipitated by saturation with ammonium sulphate. This follows the procedure of Szent-Gyorgyi (1931). The precipitated enzyme was redissolved in 0.4M sodium chloride buffered to pH 6.0 and showed negligible loss of activity.

V. THE OXIDIZING SYSTEM OF APPLE

Further work on the oxidizing system of apple has been limited by the difficulty of obtaining material of uniformly high activity. However, the investigations have generally confirmed the conclusion of Johnson and Zilva (1937) that the mechanism of oxidation is largely indirect.

An enzyme preparation was obtained from one suspension by precipitation with acetone and was dispersed in the same volume of citrate-phosphate buffer.

A further quantity of enzyme was dispersed in the acetone filtrate (from which the acetone had been boiled off), and diluted to the same volume as the original suspension. Measurements of oxidation (per cent. in five minutes) gave the following results:

Original suspension (pH 3.2)	-	-	-	-	13.9
Enzyme in citrate-phosphate buffer (pH 3.2)	-	-	-	-	0.9
Enzyme in acetone filtrate	-	-	-	-	14.8

For all except a small proportion of the oxidation, the presence of the thermostable acetone-soluble material was required as well as the enzyme. Presumably oxidation products are first formed from the soluble material, and these oxidize the ascorbic acid. The oxidizable material in the acetone filtrate may be phenolic in nature but it is doubtful if the oxidation involves a polyphenol oxidase of the usual type, which has a maximum activity at pH 6-8. The activity of the enzyme in citrate phosphate buffer of pH 3.2 was not affected by the addition of 0.1 per cent. pyrogallol.

Typical polyphenol oxidases appear to be still present, if inactive in the original suspension. When the enzyme was dispersed in citrate phosphate buffer of pH 7, the addition of 0.1 per cent. pyrogallol definitely promoted the oxidation of ascorbic acid. The results are given as per cent. oxidation in five minutes.

Enzyme in citrate-phosphate buffer (pH 7)	-	-	-	1.3
Enzyme + 0.1 per cent. pyrogallol	-	-	-	17.7

VI. DISCUSSION

The data which have been obtained both in this and the previous paper (Huelin and Stephens 1948) give some indication of the factors responsible for the varying stability of ascorbic acid in different fruits and vegetables. Ascorbic acid has been found particularly stable in onion and rose hip tissue, and in orange juice. Onion tissue has only slight oxidase activity and also contains "protective" substances which reduce copper catalysis to a very low level. Rose hip tissue has negligible oxidase activity and also contains "protective" substances. In this tissue the low pH and very high concentration of ascorbic acid (about 500-1,000mg./100g.) also tend to reduce copper catalysis. The stability of ascorbic acid in orange juice is due to similar factors. Orange juice has a comparatively high level of ascorbic acid (50-80mg./100ml.), but not as high as rose hip.

Pulped tomato tissue appears to have little oxidase activity, but offers little protection against copper catalysis. The avoidance of copper contamination in tomato products is particularly desirable.

Retention of ascorbic acid in apple products is usually very poor. Oxidase activity is very variable, but generally quite appreciable. Pressed apple juice is often completely devoid of ascorbic acid. Copper catalysis is of less importance, due to the low pH.

VII. ACKNOWLEDGMENT

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VIII. REFERENCES

- HACKNEY, F. M. V. (1946).—*Nature* 158: 133.
HUELIN, F. E., and STEPHENS, I. M. (1948).—*Aust. J. Sci. Res.* 1: 50.
JOHNSON, S. W., and ZILVA, S. S. (1937).—*Biochem. J.* 31: 438.
LOVETT-JANISON, P. L., and NELSON, J. M. (1940).—*J. Amer. Chem. Soc.* 62: 1409.
MEIKLEJOHN, G. T., and STEWART, C. P. (1941).—*Biochem. J.* 35: 755.
POWERS, W. H., LEWIS, S., and DAWSON, C. R. (1944).—*J. Gen. Physiol.* 27: 167.
STOTZ, E., HARRER, C. J., and KING, C. G. (1937).—*J. Biol. Chem.* 119: 511.
SZENT-GYORGYI, A. (1931).—*Ibid.* 90: 385.
TADOKORO, T., and TAKASUGI, N. (1939).—*J. Chem. Soc. Japan* 60: 188.
TAUBER, H., KLEINER, I. S., and MISHKIND, D. (1935).—*J. Biol. Chem.* 110: 211.

THE EFFECT OF COLOUR ON THE NUMBERS OF HOUSEFLIES RESTING ON PAINTED SURFACES

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Summary

A simple technique for determining the preference of houseflies for surfaces of various colours is described. This consists of liberating adults into a Peet-Grady testing chamber fitted with movable coloured corners. The numbers of flies resting on the coloured corners are recorded at intervals to provide adequate data for comparison.

The ascending order of preference for the particular colours tested was white and sky blue (equal), light grey, green, yellow and medium grey (equal), dusky blue, and red. From measurements of the visual reflectances of the various colours it was found that the order of preference could be explained largely by a reaction to the intensity of the light reflected by the coloured surfaces, darker surfaces being preferred to lighter surfaces.

I. INTRODUCTION

Colour perception in insects has been reviewed extensively by Weiss (1943, 1944, 1946), who points out that most of the available information indicates that light intensity is more important than wavelength in producing reactions. However, von Frisch and others have shown that, for bees at least, there is a definite wavelength discrimination (Wigglesworth 1939).

The present tests were initiated when a proposal was made during the war that army kitchens and hospitals should be painted dark blue to repel flies. While it is well known that the common bush fly, *Musca vetustissima*, will not enter darkened rooms, the reaction of the housefly, *M. domestica*, is quite different. Thus there is a general trend in the evidence of Freeborn and Berry (1935) and Atkeson *et al.* (1943), in experiments carried out in dairy barns, that flies (mainly houseflies) preferred darker to lighter colours. However, because of the absence of precise experimental control and the lack of adequate physical data on the amount of light reflected by the various colours, it was considered desirable to carry out tests paying attention to these factors.

II. METHODS

(a) Apparatus

The tests were carried out in a standard 6 ft. by 6 ft. by 6 ft. white lacquered Peet-Grady testing chamber (Anon. 1943). Into each of the four corners of the chamber was fitted a coloured, movable, plywood corner. Each plywood corner consisted of two 5 ft. 11 in. by 2 ft. 3 in. pieces hinged together so that they

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would stand up on their shorter ends at right angles to one another. During a test, these hinged pieces rested on a 2 ft. 4 in. by 2 ft. 4 in. piece of plywood placed on the floor in one corner of the chamber and were roofed by a similar piece of plywood. By this means, from one to four colours could be exposed simultaneously in the four corners of the chamber. Each false corner was separated from its neighbour by an 18 in. strip of white chamber wall.

The paints used were selected from a range of full gloss, lacquer finish colours obtained from a commercial firm. They were applied with a spray gun after appropriate undercoats.

The chamber was illuminated by a light bulb set in a reflector and shining in through a square glass panel in the centre of the roof. Normally a 200-watt frosted bulb was used, but in some experiments the light intensity was varied by inserting other bulbs. Although the light in this position provided equal illumination for each of the four coloured corners, the intensity of illumination varied from place to place on each corner. Thus the roof and upper portion of each corner was in shadow, while the remainder was directly illuminated, but situated at varying distances from the source of light. However, since each corner had an identical distribution of light from the bulb, this variation in light intensity over any one corner should not introduce any irregularities when comparing the relative attractiveness of colours.

The temperature of the chamber was thermostatically controlled, the majority of tests being carried out at $75^{\circ} \pm 1^{\circ}\text{F}$.

(b) *Flies Used*

The test insects were adult houseflies (*Musca domestica* L.), bred in general accordance with the standard Peet-Grady technique (Anon. 1943). They were fed on a mixture of equal parts of milk and water and were usually tested when three to seven days old. Most batches of flies consisted of approximately equal numbers of males and females. However, in one test, females alone were used.

(c) *Test Procedure*

Four hundred to five hundred flies were liberated in the chamber in which corners of the desired colours had been placed. The operator sat on a stool in the centre of the chamber and disturbed all flies from each of the four corners in rotation and then from the glass panel under the light. After an interval of three minutes to allow the flies to settle, the numbers of flies on each coloured corner were counted as rapidly as possible, starting with the corner which had been disturbed first. Following this count, the flies were again disturbed and counted after settling, on this occasion the corner first disturbed being the one adjacent and clockwise to the one first disturbed previously. When each of the four corners had been the first to be disturbed, a cycle of four counts was complete. At least four repetitions of each cycle of four counts were usually carried out with each batch of flies to provide sufficient data for statistical analysis.

The above cyclical procedure was necessary to compensate for the fact that the corner last to be disturbed for any one count always had fewer flies resting on it than would have been expected from the counts when it was not disturbed last. The validity of the cyclical method of counting is discussed later.

In one series of experiments, a cycle of counts taken inside the chamber in the fashion just mentioned was alternated with a cycle taken by an operator through glass panels in opposite sides of the chamber. During the outside cycle of counts the flies were not disturbed but, as before, counts were taken at three-minute intervals. This series of experiments was designed to detect the presence of any "self-preservation" reaction which might cause the flies to react differently to coloured panels when disturbed than when allowed free choice to settle and move without disturbance.

(d) Colours Tested

Table 1 contains information* on the colours tested. Plywood painted with each colour and illuminated by illuminant A (light from a gas-filled tungsten lamp operating at a colour temperature of 2,840°K.) was matched to the nearest shade in the Munsell Book of Colour. From the Munsell notation the ISCC-NBS name was derived (Judd and Kelly 1939), and the I.C.I. trichromatic coefficients for illuminant A (gas-filled light bulb) obtained (Kelly *et al.* 1943). The visual reflectances were measured with illuminant A, incident normally on the sample and viewed at 45° to the normal. This source of light is the standard equivalent of the 200-watt bulb used to illuminate the chamber.

TABLE 1
DESCRIPTION OF THE COLOURS TESTED

Name Used	Munsell Notation	ISCC-NBS Name	I.C.I. Trichromatic Coefficients			Visual Reflectance (=Y)
			x	y	z	
White	10Y8/2	Pale yellow-green	0.460	0.422	0.118	0.68
Sky blue	5BG7/2	Pale blue-green	0.421	0.420	0.159	0.50
Dusky blue	5B3/4	Dusky blue	0.333	0.390	0.277	0.04
Green	10GY6/6	Strong yellowish-green	0.419	0.482	0.099	0.31
Yellow	10YR7/10	Moderate yellowish-orange	0.545	0.431	0.024	0.37
Red	5R4/14	Vivid red	0.641	0.331	0.028	0.16
Light grey	N7/	Light grey	0.446	0.408	0.146	0.45
Medium grey	N5/	Medium grey	0.446	0.407	0.147	0.23

*Supplied by the Division of Physics, C.S.I.R.

Light grey was prepared by mixing one part of black paint with eleven parts of white, while medium grey was a mixture of one part of black and three parts of white.

(e) *Analysis of Results*

The relative attractiveness of colours was compared on the basis of the ratio of the numbers of flies on the panels in question, these numbers being summated over the four phases of a cycle. Logarithms of ratios for replicate cycles were used for tests of significance. When a number of cycles was involved the mean value for a colour ratio was calculated as the antilog of the mean log of the ratios of each cycle.

III. RESULTS

A. STANDARDIZATION EXPERIMENTS

(a) *Symmetry of Method of Counting*

In order to test for any possible asymmetry in the test chamber or in the method of taking counts, one pair of diagonally opposite corners was set up with white plywood and the other with dusky blue plywood. One hundred and six cycles, each of four counts, were then made, as described earlier, ten different batches of flies being used. Between tests with each batch of flies both the painted corners and the chamber were wiped clean with a moist cloth. The results obtained are shown in Table 2.

TABLE 2
COUNTS OF FLIES RESTING ON WHITE AND DUSKY BLUE CORNERS

Corner	1	2	3	4
Colour	Blue	White	Blue	White
No. of flies	24,389	3,797	24,715	3,741

There was no significant difference in the ratios of the numbers of flies resting on the two blue or the two white corners. This indicates that there is no asymmetry of the chamber, and that no asymmetry is introduced by the method of counting. Hence we can conclude that it is unnecessary to have more than one corner of any colour in order to obtain reliable results. Absence of asymmetry due to counting was further substantiated when the ratio of blue to white was compared for the first and second halves of each cycle of four counts. It was found that there was no significant difference, indicating that the ratio could be established satisfactorily by half a cycle when only two colours were compared. It is clear, however, that a complete cycle is necessary when four colours are compared simultaneously.

(b) *Effect of Recent Painting*

As a regular precaution, panels were allowed to age for at least five days after painting, before use. However, on one occasion (see Table 3) a test was

carried out to compare the attractiveness of a surface painted the day before with one which had been painted five days previously. These two corners were again compared on three successive days. The more recently painted panel had fewer flies (not significant) resting on it on the first and second days after painting than the other panel. However, by the third day any trend that there may have been had disappeared. The five-day minimum ageing period adopted as a standard was therefore considered ample.

TABLE 3

COMPARISON OF THE NUMBERS OF FLIES RESTING ON PANELS ALLOWED TO AGE FOR DIFFERENT TIMES AFTER PAINTING WITH DUSKY BLUE

Panel Painted	Date Tested			
	22.v.43	23.v.43	24.v.43	25.v.43
17.v.43	954	2,035	1,309	1,047
21.v.43	920	1,795	1,478	1,060

(c) *Effect of Light Intensity and Temperature*

Several tests were carried out using 150- and 60-watt globes in place of the 200-watt globe. The results were somewhat conflicting, although the bulk of the evidence indicated that alteration of light intensity over this range did not affect the ratios obtained. Similarly, several tests were carried out at 70°, 80°, and 85°F. instead of at 75°F. In some tests the ratios were lower at a higher temperature than at a lower one, but this trend was not consistent. There was no occasion on which alteration in light intensity or temperature altered the relative order of attractiveness of the colours.

(d) *Effect of Sex of Fly*

On one occasion the chamber was populated with female flies only, white and red panels being compared. The white : red ratio from eight cycles was 1 : 6.2 which is very similar to the 1 : 6.9 ratio obtained from different cultures using both males and females. There is no indication, therefore, that the sex of the fly influences its response to colour.

B. RESULTS OF COLOUR COMPARISONS

Unless specifically mentioned, all tests in this section were carried out at 75°F. using a 200-watt light bulb. Table 4 shows the ratios obtained in tests when two colours only were compared. Owing to the variation in ratios from day to day and from culture to culture, it is only satisfactory to use, for an accurate comparison of various colours, the ratios obtained when these colours are directly compared. However, the ratios given in Table 4 do indicate quite well the relative attractiveness of the various colours.

TABLE 4

RATIOS OBTAINED FROM COMPARISON OF TWO COLOURS ONLY

Colour	White : Colour = 1 :	Green : Colour = 1 :	Yellow : Colour = 1 :
Sky blue	1		
Light grey	(1.5) *		
Green	2.0		
Yellow	2.3	1.2	
Medium grey	(2.4) *		
Dusky blue	6.2		
Red	6.9	4.3	5.3

*Derived from comparison when other colours were present.

(a) *Sky Blue*

Sky blue was compared only with white, 25 cycles giving total counts of 5,259 flies on white and 5,290 on sky blue, the mean ratio not departing significantly from 1. The white : sky blue ratios for individual cycles varied from 1 : 0.76 to 1 : 1.4

(b) *Light Grey*

Light grey was compared with white on a number of occasions, but each time in the presence of other colours. The white : light grey ratios recorded were 1 : 1.5 (12 cycles in the presence of yellow and red), 1 : 1.5 (4 cycles with medium grey and red), and 1 : 2.2 (4 cycles with medium grey and yellow).

The light grey : yellow ratios obtained from these comparisons were both 1 : 1.4 and there is confirmatory evidence from tests at 80°F. that light grey is less attractive than yellow.

No comparisons with green were carried out at 75°F. but, at 80°F., the average light grey : green ratio of 26 cycles in the presence of white and yellow was 1 : 1.8 (and of light grey to yellow, 1 : 2.0). Light grey is, therefore, less attractive than green, but more attractive than white.

(c) *Green*

The white : green ratio for 13 single-comparison cycles was 1 : 2.0, while the average ratio from 26 cycles in the presence of yellow and red was 1 : 1.4. The green : yellow ratio from these tests was 1 : 1.4, and the green : red ratio 1 : 4.8. This green : red ratio was of a similar order also in other tests, for instance, 1 : 4.3 for 19 cycles of these two colours alone and 1 : 4.7 for 18 cycles in the presence of yellow.

The green : yellow ratio was further investigated with 23 cycles in which these two colours alone were compared (1 : 1.2) and 18 cycles in which red was also present (1 : 1.3). Green is, therefore, less attractive than yellow.

(d) Yellow

The white : yellow ratio for 8 single-comparison cycles was 1 : 2.3. Other ratios obtained were 1 : 2.0 (26 cycles with green and red), 1 : 2.0 (12 cycles with light grey and red) and 1 : 3.1 (4 cycles with light and medium grey).

In addition to comparisons already mentioned, yellow has been compared with medium grey and red. The yellow : red ratios obtained were 1 : 5.3 (14 single-comparison cycles), 1 : 3.6 (26 cycles with white and green), 1 : 3.7 (18 cycles with green), and 1 : 5.0 (12 cycles with white and light grey). The yellow : medium grey ratios obtained were 1 : 0.9 (8 cycles with white and green), and 1 : 1.1 (13 cycles with white and light grey). There was no significant difference in the relative attractiveness of these two colours.

(e) Medium Grey

The white : medium grey ratios obtained were 1 : 2.4 (4 cycles with light grey and red) and 1 : 3.2 (4 cycles with light grey and yellow).

Medium grey has already been shown to be similar in attractiveness to yellow; it is less attractive than red, and more attractive than green (green : medium grey = 1 : 1.25 for 8 cycles with white and yellow).

(f) Dusky Blue

This paint had been selected by a manufacturer as a fly-repellant blue paint. A total of 72 cycles of comparisons with white was performed, giving an average white : blue ratio of 1 : 6.2; 5,659 flies being counted on white corners and 34,451 on dusky blue corners. These tests were carried out over quite a long period of time and involved many different cultures of flies. It is of interest to note that the lowest ratio recorded for a cycle was 1 : 3.5 and the highest 1 : 15.3. Low or high ratios appeared to be associated with particular batches of flies, but no explanation was found for their varying behaviour.

(g) Red

The white : red ratio for 25 single-comparison cycles was 1 : 6.9. Values also obtained for this ratio were 1 : 6.2 (26 cycles with green and yellow) and 1 : 9.0 (20 cycles with light grey and yellow). No comparisons were made of dusky blue and red, but it seems reasonable to assume that these colours both have the same order of attractiveness.

It is apparent from the foregoing results that the ratios may vary considerably at different times. Thus, when the attractiveness of two colours is close, e.g. sky blue and white, medium grey and yellow, or even occasionally with green and yellow, either one or other colour may record the greater number of flies. When the attractiveness is quite different the ratios obtained with different fly cultures may vary greatly, e.g. the fourfold variation for white: dusky blue, although the order of attractiveness of the colours remains the same.

A series of tests was set up to determine the quantitative accuracy of ratios obtained for various colour combinations with the same batch of flies. Four cycles of counts were carried out with each of the following pair comparisons: green *v.* red, yellow *v.* red, and yellow *v.* green. The results are shown in Table 5.

TABLE 5
RATIOS OBTAINED FROM COLOUR TESTS ON THE SAME BATCH OF FLIES

Green : Red	= 1 : 5.87
Yellow : Red	= 1 : 6.28
Yellow : Green	= 1 : 1.07

The observed ratios are exactly the same as those obtained by calculation. For example, the observed green : yellow ratio is the same as that calculated from the green : red and yellow : red ratios. This suggests that for a single batch of flies there is a fixed quantitative difference in response to the colours. It is interesting to note that in this test the green corners had more flies on them than the yellow corners.

As a means of testing the stability of ratios in a changing environment, 22 cycles of green, yellow, and red were run, in which the fourth panel was white, and these were interspersed with 18 cycles in which the fourth panel was another red one. The values obtained for the three panels which remained constant throughout are shown in Table 6, there being no significant difference in the two sets of ratios. In other words, the relative attractiveness of the three colours tested was not influenced by the presence of a varying fourth colour.

TABLE 6
RATIOS OBTAINED FROM COLOUR TESTS

Fourth Colour	Ratios
White	Green : Yellow : Red = 1 : 1.4 : 5.1
Red	Green : Yellow : Red = 1 : 1.3 : 4.9

In the counts carried out inside the chamber it was thought that there might be, superimposed on a colour preference, some sort of "self-preservation" response whereby flies which were endeavouring to escape from a disturbing influence would rest preferentially on darker colours. To test this possibility, counts inside the chamber were alternated with counts taken from outside through windows in opposite sides of the chamber. The results are shown in Table 7. There is a significant difference in the relative numbers of flies on the various colours when the observer counts from inside and outside the chamber, the effect of the observer in the chamber, taking white as reference, being given by the following:

<i>White</i>	<i>Light Grey</i>	<i>Green</i>	<i>Dark Grey</i>	<i>Yellow</i>
1.000	1.023	1.042	1.119	1.127

Subject to the remote possibility of observer bias (omitting to count all the flies on the darker panels) one can state that the presence of the observer in the chamber tends to make the darker colours relatively more attractive. However, because of the correlation of the effect of the observer with the attractiveness of the colours, the ranking remains the same. This is the only relevant issue,

TABLE 7

COMPARISON OF RATIOS OBTAINED FROM COUNTS OUTSIDE AND INSIDE THE CHAMBER

	White	Light Grey	Green	Dark Grey	Yellow	No. of Cycles
Outside	1	1.2	1.7		2.0	20
Inside	1	1.1	1.9		2.2	28
Inside	1	1.8		2.8	3.2	13
Outside	1	1.6		2.6	2.9	9
Inside	1		2.3	2.8	2.5	8
Outside	1		2.3	2.5	2.3	8

since the general conclusions reached from the data already presented are thus valid under the two sets of circumstances, either of which may be encountered by the fly during the selection of a colour upon which to settle.

IV. DISCUSSION

It has been clearly established that houseflies have a definite order of preference for surfaces of various colours. Such a preference may be either for the colour of the surface, or the intensity of light reflected, or a combination of both factors.

If the ratios obtained when the colours were compared with white (Table 4) are plotted against the visual reflectances of these colours (Table 1), it can be seen (Fig. 1) that there is a good correlation between the amount of light reflected by the painted surface and its attractiveness to flies, the lighter colours being less attractive than the darker colours. In Figure 1 there are several irregularities in the order of the colours, but these are probably due in part to the fact that the ratios were established on different cultures of flies and are, therefore, not strictly comparable. In addition, without knowing the curve for the relative stimulative efficiency of different wavelengths for the housefly retina, it is not legitimate to assume that the figures used for visual reflectance are true values for the relative intensities perceived by the flies.

Indeed there is good evidence that insects are less sensitive to the red end, and more sensitive to the violet end, of the spectrum than the human eye (see Wigglesworth 1939). This might well account for the fact that both yellow and red gave ratios suggesting that they appeared less bright to the flies than one would have expected from their visual reflectances. The fact that reduction in

intensity of illumination did not alter the relative order of attractiveness of colours suggests that the optimum intensity of reflected light must be zero, or close to it. No clear evidence in these tests was obtained of a response to wavelength.

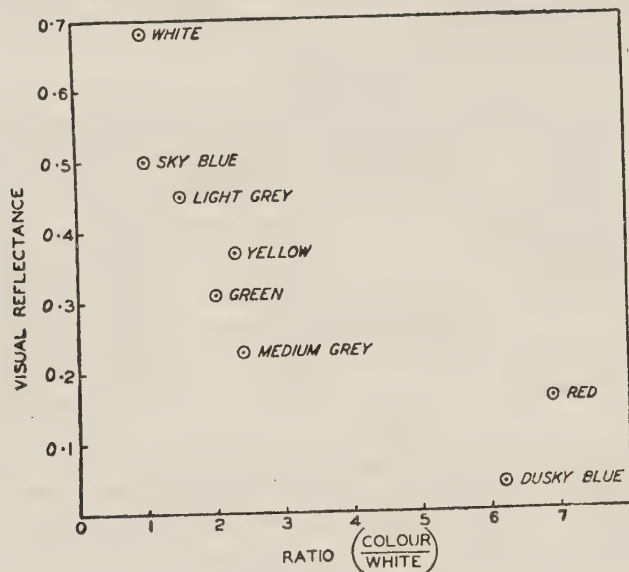


Fig. 1.—Graph showing the relationship between visual reflectance and attractiveness of colours to houseflies.

Considering the use of colours to deter flies from entering rooms, it seems highly improbable from general observations that even the least attractive colours will have an overriding effect on the powerful chemotropic stimuli guiding houseflies to an attractive odour. Once the flies have fed or ceased to respond to the odour it is possible that the colour of their surroundings may affect the numbers which remain or leave. Lighter shades of colours are, therefore, to be preferred to darker shades. However, the effectiveness of residual deposits of poisons, such as DDT, suggests that colour will only be called on to play, at the best, an accessory part in affecting the accumulation of flies in rooms. Dark colours, for instance, might well be used to attract houseflies to surfaces treated with DDT, thereby restricting the areas which it is necessary to treat.

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VI. REFERENCES

- ANON. (1943).—Peet-Grady method. *Soap Sanit. Chem.*, Blue Book.
- ATKESON, F. W., SHAW, A. O., SMITH, R. C., and BORGMAN, A. R. (1943).—Some investigations of fly control in dairy barns. *J. Dairy Sci.* 26: 219-32.
- FREEBORN, S. B., and BERRY, L. B. (1935).—Colour preferences of the housefly *Musca domestica* L. *J. Econ. Ent.* 28: 913-6.
- JUDD, D. B., and KELLY, K. L. (1939).—Method of designating colours. *J. Res. Nat. Bur. Stand.* 23: 355-86.
- KELLY, K. L., GIBSON, K. S., and NICKERSON, D. (1943).—Tristimulus specification of the Munsell book of colour from spectrophotometric measurements. *J. Opt. Soc. Amer.* 33: 355-76.
- WEISS, H. B. (1943).—Colour perception in insects. *J. Econ. Ent.* 36: 1-17.
- (1944).—Insect responses to colours. *J. N.Y. Ent. Soc.* 52: 267-71.
- (1946).—Insects and the spectrum. *Ibid.* 54: 17-30.
- WIGGLESWORTH, V. B., 1939.—“The Principles of Insect Physiology.” (Methuen & Co. Ltd.: London.)

THE EFFECT OF STARVATION ON THE SUSCEPTIBILITY OF HOUSE-FLIES TO PYRETHRUM SPRAYS

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Summary

Pyrethrum sprays produced higher mortalities among laboratory-reared houseflies which were starved for periods of 1 to 6 hours immediately prior to exposure than among flies which were given continuous access to food before testing. Within this range of starvation time, mortality increased, but not uniformly, with starvation time. The factors involved in the relationship of mortality to starvation time have been examined. It was found that the dosages of spray retained by individual flies after exposure varied with starvation time, and an analysis is given of the principal factors responsible for this variation.

Methods used for drawing samples of houseflies from a population for insecticidal tests are discussed, and attention is drawn to the possibility that starvation of the flies caused by the interruptions to their feeding routine during sampling and pre-test treatment may introduce unnecessary variability between samples.

A method for increasing the susceptibility of houseflies to pyrethrum sprays, without affecting the rate of change of fly mortality with pyrethrum concentration, is suggested.

I. INTRODUCTION

When houseflies (*Musca domestica* L.) are reared for use in fly spray tests, their susceptibility to pyrethrum sprays is not affected by starvation for short periods (which, in complete darkness, may exceed 12 hours), provided they are given access to food for a sufficient time, immediately prior to testing, to ensure that all have had a recent feed when sprayed.

If the pre-test feed, following starvation, is omitted, a pyrethrum spray will produce a higher mortality of the starved flies than of flies which have been fed continuously. This is clearly shown in Figure 1 by the relative positions of the concentration-mortality regression lines for flies starved for 2 hours immediately prior to testing, and flies fed continuously. The points plotted represent mean probit values for the mortalities obtained in 6 replications at each concentration using about 250 flies of each sex per test. The method of testing used was the Peet-Grady method (Anon. 1943, 1946).

The factors involved in this apparent increase in susceptibility of flies to pyrethrins, caused by starvation, are the main subject of this investigation. All experiments recorded herein were conducted at $24^{\circ}\pm 1^{\circ}\text{C}$, and during the various starvation treatments, the flies were continuously and uniformly illuminated.

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II. EXPERIMENTAL PROCEDURE AND RESULTS

(a) *The Effect of Pre-Test Starvation on Mortality of Flies treated with Pyrethrins*

Several cultures of adult houseflies were prepared for spray tests by the Peet-Grady method. A culture consisted of groups of 300-500 flies which had

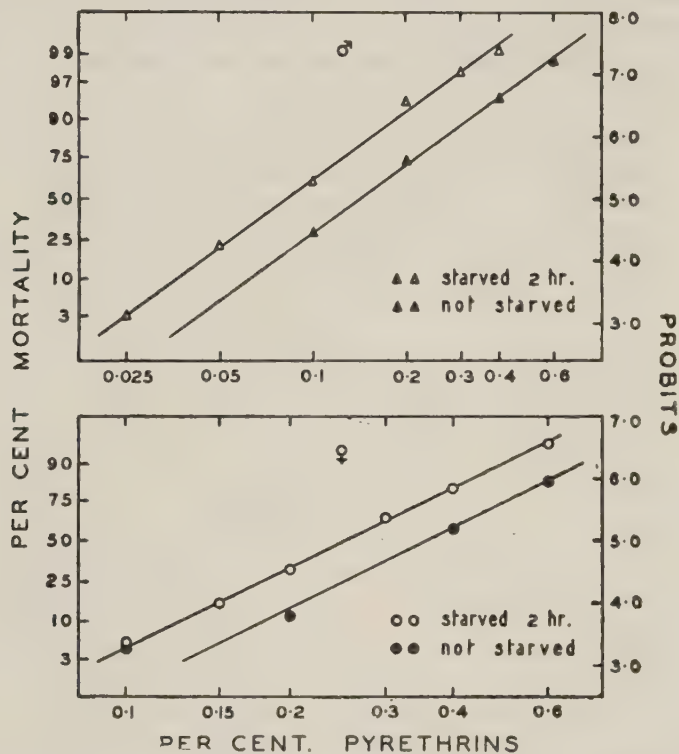


Fig. 1.—Mortality plotted against concentration of pyrethrins for flies starved for 2 hours, and flies fed continuously.

emerged from randomized puparia obtained from one larval culture. Approximately equal numbers of males and females were present in each group. When used for testing, the flies were 3-5 days old.

Up to the day of testing, the flies were fed continuously on a 5 per cent. mixture of powdered milk extract and water, which was renewed twice daily. On the day of testing, one group of flies from each culture was supplied with food right up to the time of liberation in the testing chamber. Other test groups from the same cultures were denied access to food for periods of 1, 2, 3, 4, and 6 hours immediately preceding the tests. Flies starved for periods greater than 6 hours were not used because, in preliminary tests, it was found that some began to show signs of sluggishness and weakness after these longer periods of starvation.

Each group was sprayed in the normal Peet-Grady manner with 12 ml. of a kerosene based spray containing either 0.1 or 0.2 per cent. w/v of total pyrethrins. In computing mortalities, 24 hours after testing, males and females were counted separately. These tests were replicated seven times.

Curves relating kill, on a probit scale, to starvation time are shown in Figure 2 for males sprayed with the 0.1 per cent. solution, and for males and females exposed to the 0.2 per cent. spray. The mortalities of female flies sprayed with 0.1 per cent. of pyrethrins were at too low a level (less than 8 per cent.) to be of use in the assessment of the effects of pre-test starvation.

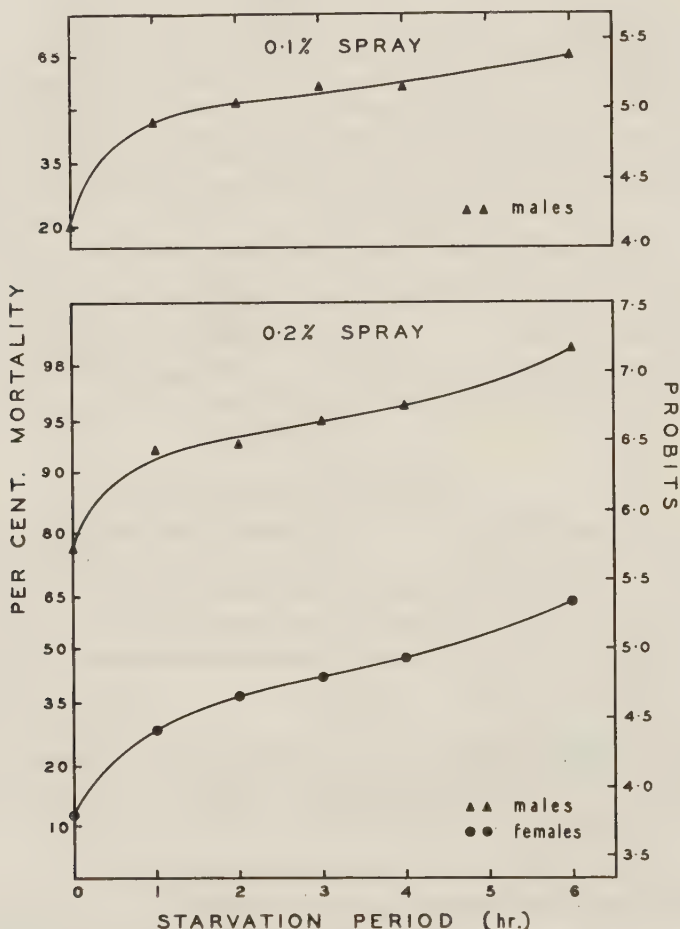


Fig. 2.—Mortality of flies, sprayed with 0.1 and 0.2 per cent. of pyrethrins, plotted against pre-test starvation time.

If the sole effect of starvation in these tests were to change the susceptibility of the flies, then the shapes of the curves do not accord with what might be conjectured as reasonable, such as an initial uniform increase followed possibly by a

greater rate of increase. It would therefore appear likely that when houseflies are starved for various periods prior to testing, the mortality produced by a pyrethrum spray in Peet-Grady tests is not governed solely by the effect of starvation on the susceptibility of the flies to the toxic action of pyrethrins.

The writer has described in detail an Individual-Dosage method designed for testing pyrethrum adjuvants (to be published later). This method, which involves the use of a micro-burette for applying measured dosages of pyrethrum solutions to individual flies, was adapted in the following manner to tests with fed and starved flies:

Ten males and ten females were collected, singly and at random, from a culture of 3-5-day-old flies which had been supplied with food continuously. Each fly was immediately given a standard dosage of pyrethrum solution, applied to the mesonotum, and liberated into an observation cage. The volume of each dosage was 0.106 c.mm., and the weights of pyrethrins applied to males and females were 0.40 and 0.80 μ g. respectively. The food was removed from the

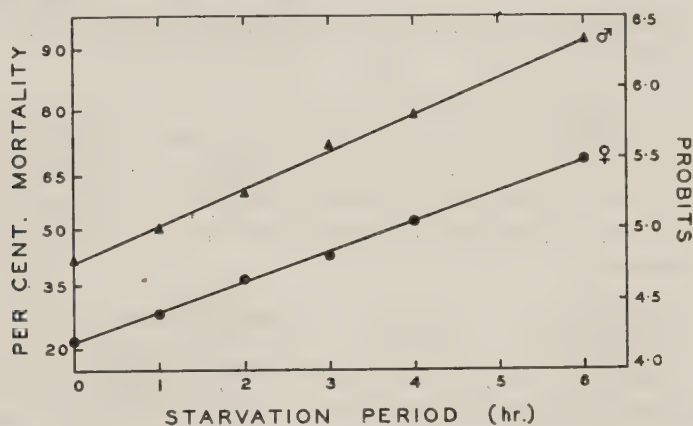


Fig. 3.—The effect of pre-test starvation on the susceptibility of flies to the toxic action of pyrethrins.

culture cage and the above procedure repeated when the remaining flies had been starved for 1, 2, 3, 4, and 6 hours. A small pad of cotton wool moistened with a 10 per cent. aqueous solution of cane sugar was placed in each observation cage to serve as food and moisture for flies which subsequently recovered from the effect of the pyrethrins. Mortality counts of male and female flies were made 24 hours after each test. Twelve replications of this series of tests gave the mean results shown graphically in Figure 3, in which mortality, on a probit scale, is plotted against starvation time.

Figure 3 shows a marked difference between the susceptibilities of male and female houseflies to pyrethrins. Although the weight of pyrethrins applied to males was only one-half of that applied to females, in all groups of flies higher mortalities were recorded among males than among females.

The shapes of the curves, which may be regarded as straight lines, are quite distinct from those obtained in Peet-Grady tests where the dosages received by individual flies were not controlled (cf. Fig. 2). This difference is particularly noticeable in the range 0.2 hours of starvation time. The dissimilarity between the two sets of curves strongly supports the view, expressed earlier, that factors other than the susceptibility of flies to pyrethrum poisoning are involved in the effect of pre-test starvation on the mortality of flies sprayed with pyrethrins. Since the main difference between the two methods of testing was that the dosages received by individual flies were kept constant in the uniform-dosage tests, and were not controlled in the Peet-Grady tests, the indications are that pre-test starvation affects the dosages received by the flies when exposed to pyrethrum sprays.

(b) The Influence of Pre-Test Starvation on the Dosages retained by Flies after Exposure to Pyrethrum Sprays

In order to provide evidence as to whether pre-test starvation influences dosage in Peet-Grady tests, colorimetric determinations of the amounts of spray on the flies after exposure were carried out.

A strong filtered solution of the dye Sudan III in kerosene was used to prepare pyrethrum sprays containing 0.1 and 0.2 per cent. w/v of total pyrethrins. Preliminary tests with a dyed spray showed that the dye itself had no effect on the toxicity of the spray or the behaviour of the flies when sprayed. The dye was completely recoverable from the bodies of the flies by successive extractions with small volumes of kerosene.

The dyed sprays were used in tests on houseflies, continuously fed, and starved for periods of 1, 2, 3, 4, and 6 hours preceding the tests. At the end of the exposure period in each test, 100 males and 100 females were collected from the flies paralyzed on the floor of the chamber, and placed in small vials. A small quantity, approximately 5 ml., of kerosene was added to each vial. The remainder of the flies in each test were collected in the normal manner and kept for mortality counts 24 hours later.

During the exposure of the flies to the dyed spray it was seen that a considerable quantity of insecticidal liquid was splashed from the bodies of the flies when they hit the walls of the testing chamber, and that a further amount was removed while the knocked-down flies were buzzing on the floor. Thus more was received by the flies during exposure than was retained afterwards. This phenomenon has also been noted by David (1945).

The kerosene was drained from each group of dyed flies into small clean vials. The flies were then washed with 3 successive 5 ml. lots of kerosene, each washing being drained into the vial. These 4 extractions were sufficient to remove all visible traces of dye from the flies. A fifth wash was then given and the volume of the extract made up to 25 ml. with kerosene.

Suitable standard solutions containing known weights of pyrethrins were prepared by diluting the dyed sprays with kerosene. The extracts from the groups of dyed flies were then compared with the standard solutions, using a Lange photoelectric colorimeter. From these colorimetric comparisons the mean weights of pyrethrins retained by the flies in the various pre-test starvation groups were calculated.

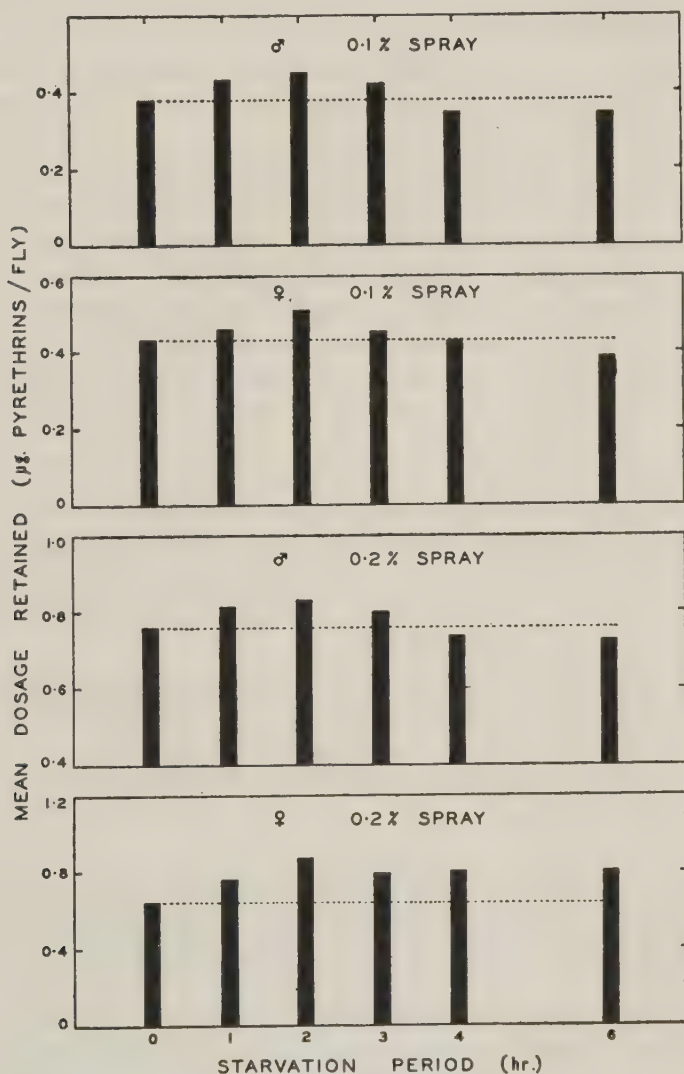


Fig. 4.—Effect of pre-test starvation on the dosages retained by flies after exposure to 0.1 and 0.2 per cent. pyrethrum sprays.

The mean weight of pyrethrins retained by continuously-fed flies in four Peet-Grady tests with the 0.1 per cent. spray was found to be 0.378 $\mu\text{g.}$ for

males and 0.431 μg . for females. In three tests with the 0.2 per cent. spray the corresponding weights were 0.759 and 0.640 μg . Dosages retained by starved and fed flies are given in Figure 4, which shows that the mean dosages retained by flies of both sexes reached a maximum for flies starved for 2 hours and decreased as starvation time increased beyond this interval. Male flies in the 4- and 6-hour groups actually retained less than continuously-fed males, and the same applies also to females sprayed with 0.1 per cent. of pyrethrins.

In considering the relationship between dosages received and dosages retained, if the amounts shed are not proportional to the total amounts received, the amounts received and retained will also not be proportional. However, the orders of ranking of the starvation periods with respect to total amounts received and amounts retained are likely to be the same. Further, the toxic effect of the changing dosage to which an individual fly is subjected before and after knock-down is probably not greatly different from the effect of a constant dosage equal to the dosage retained after exposure. If these arguments are valid, the principal reason for the dissimilarity between the curves for the Peet-Grady tests and the lines given by the uniform-dosage method must be the variation in mean effective dosage received by the flies in the different starvation-period groups.

(c) *Factors influencing the Variation in Dosage Received with Starvation Time*

Some spray will settle on flies which are on the walls and floor of the testing chamber, but the amount acquired per unit time will be much higher when the flies are flying and will almost certainly increase with the speed of flight. Accordingly, the following may be considered as factors determining the dosage and the variation in dosage between groups of flies:

- (1) The rate of knock-down, or inversely, the mean interval before knock-down.
- (2) The proportion of unparalyzed flies which are in flight.
- (3) The speed of flight.

Although logically (1) and (2) should be grouped together as determining the amount of flying in successive intervals of time, it is more convenient to deal with (2) and (3) together under the descriptive term "fly activity."

(i) *Fly Activity during Exposure to Pyrethrum Sprays.*—The measurement of activity presents a difficult problem with the large numbers of insects used in Peet-Grady tests, and with the facilities available it was not possible to obtain absolute activity values. However, it was found that the differences between groups of fed and starved flies, in the proportion of unparalyzed flies in flight and in the speed with which they flew, were quite appreciable. In each Peet-Grady test the following observations were therefore made:

At each of several instants during the first minute after spraying, which took about 55 seconds, an estimate was made of the per cent. of unparalyzed flies in flight. It was found that there was very little variation in these individual estimates which were averaged to give the mean estimated per cent. of unparalyzed flies in flight during the first minute after spraying.

A rating was allotted, in each test on starved flies, for the mean speed of flight during the first minute after spraying, compared with that shown by continuously-fed flies in the same series of tests, according to the arbitrary scale given in Table 1.

TABLE 1
RATING SCALE FOR MEAN SPEED OF FLIGHT

Mean Speed of Flight of Starved Flies compared with Fed Flies	Rating
Slightly less	-1
Equal	0
Slightly greater	+1
Greater	+2
Much greater	+3

The broad differences in mean speed of flight upon which this scale was based were easily discernible even by untrained observers. As a further precaution against personal bias in the estimations of fly activity, the identity of the starvation period in each test was withheld from the observer.

Mean results obtained by these methods in tests with fed and starved houseflies are presented in Table 2. As indicated by the fractional mean values given, the rating for mean speed of flight allotted to a starvation group was not the same in each replication, but in none of the groups did the variation in individual ratings exceed 1 point on the rating scale.

At the time of exposure, the sex ratio of flies used in each test was not known, and since males and females may respond differently to the stimulating action of pyrethrum sprays, there is a possibility that the sex ratio of the flies may have biased the estimates of activity. When the final counts of flies were made for mortality calculations, 24 hours after exposure, sex ratio factors were computed for each test group of flies. These factors were obtained by dividing the percentage of males in each test group by the percentage of males in the corresponding group of continuously-fed flies. The mean values for the sex ratio factors, given in Table 2, show that the differences between starvation groups in the sex ratio of flies were not excessive, and were therefore not likely to have biased greatly the activity estimates.

TABLE 2

EFFECT OF PRE-TEST STARVATION ON FLY ACTIVITY DURING EXPOSURE TO PYRETHRUM SPRAYS

Starvation Period (hours)	Mean Per Cent. of Unparalyzed Flies in Flight		Mean Speed of Flight Rating		Sex Ratio Factor	
	0.1% Spray	0.2% Spray	0.1% Spray	0.2% Spray	0.1% Spray	0.2% Spray
0 (Fed flies)	41	48	0	0	1.00	1.00
1	56	64	+1.3	+1.2	0.96	0.98
2	61	70	+2.0	+1.8	1.00	1.06
3	64	70	+2.0	+1.8	0.93	1.01
4	60	71	+2.0	+1.8	0.91	0.94
6	61	71	+2.0	+1.8	0.92	0.90

The results show that activity, as estimated during the first minute after spraying, was virtually constant in flies starved for periods of 2 to 6 hours, and that the activity of these starved flies was estimated to be much higher than in flies which had not been starved. Flies which were subjected to starvation for one hour appeared to be intermediate between these two groups.

Obviously the methods used for assessing the activity of the flies have their limitations, and it is realized that the mean values given in Table 2 are only indications, rather than precise measurements, of the effects of starvation on the activity of the flies when exposed to the pyrethrum sprays. However, it is desired to point out that as starvation time is increased from 0 to 2 hours there is a close correlation between estimated activity (Table 2) and the dosages retained by the flies (Fig. 4).

(ii) *The Rate of Knock-Down of Flies exposed to Pyrethrum Sprays.*—At intervals of 1, 2, 3, 5, and 10 minutes after the completion of spraying, i.e. at approximately 2, 3, 4, 6, and 11 minutes after spraying was commenced, rapid counts were made of the flies which were either paralyzed on the floor of the chamber, or not paralyzed, whichever was the smaller number. From these values, cumulative percentages of flies knocked down were calculated. Table 3 shows the results obtained with the 0.1 per cent. spray.

Thus starved flies were knocked-down more rapidly by the spray than flies which had been fed continuously, and the rate at which knock-down proceeded increased with starvation time. Activity appeared to be constant in the 2- to 6-hour starvation groups, and if the physical properties of the spray mists also remained unchanged during the exposure periods, one would expect the amount of spray collected to be proportional to the mean flying time and to the mean time before knock-down. Evidence for such proportionality cannot be given, for the mean amounts of spray collected were, of course, not known, and the mean times before knock-down could not be calculated because knock-downs were still incomplete at the end of the exposure periods.

TABLE 3

EFFECT OF PRE-TEST STARVATION ON THE KNOCK-DOWN OF FLIES SPRAYED WITH 0.1 PER CENT. OF PYRETHRINS

Starvation Period (hours)	Per Cent. Knock-Down at Intervals after Spraying Commenced				
	2 min.	3 min.	4 min.	6 min.	11 min.
0 (Fed flies)	15.4	36.1	57.5	74.6	90.3
1	25.6	60.4	77.9	85.6	95.8
2	40.1	68.5	80.3	88.2	95.8
3	41.7	73.9	85.8	90.7	96.8
4	53.4	78.6	88.0	93.6	97.5
6	71.1	83.2	89.8	95.2	98.7

The values given in Table 3 show that the curves of percentage knock-down plotted against time, for the various starvation periods, do not intersect. It is clear, then, that the mean time before knock-down decreased with increasing starvation time. Thus, as starvation time increased from 2 to 6 hours, the mean time before knock-down and the mean of the dosages retained after exposure by males and females decreased together. It is therefore reasonably certain that, among the flies starved for 2 to 6 hours, the reductions in dosage retained as starvation time increased were due principally to the decreases in mean flying time.

TABLE 4

EFFECT OF PRE-TEST STARVATION ON THE KNOCK-DOWN OF FLIES SPRAYED WITH 0.2 PER CENT. OF PYRETHRINS

Starvation Period (hours)	Per Cent. Knock-Down at Intervals after Spraying Commenced				
	2 min.	3 min.	4 min.	6 min.	11 min.
0 (Fed flies)	39.8	65.2	80.4	89.4	96.7
1	58.8	82.9	90.3	95.3	98.8
2	71.5	86.5	93.6	97.2	99.1
3	74.3	88.0	93.4	98.6	99.3
4	74.4	87.9	94.4	97.1	99.1
6	77.9	90.3	94.9	97.5	99.2

With the spray containing 0.2 per cent. of pyrethrins, knock-downs, as shown in Table 4, were greater and proceeded more rapidly than those obtained with the 0.1 per cent. spray (Table 3). For the starvation period range 0 to 2 hours, the relationship between rate of knock-down and starvation time was similar to that for the weaker spray. However, there was comparatively little change in rate of knock-down when starvation time was increased from 2 to 6 hours. This strongly suggests that, in these groups of flies, the rate of knock-down was very near to its upper limit. When the upper limits of fly activity and rate of knock-down are reached, one would expect the mean amount of spray collected by the flies to remain constant irrespective of further increase in starvation time.

In this regard it is of interest to note, in Figure 4, that the mean dosages retained by both male and female flies starved for 6 hours are almost identical with those retained by flies in the 4-hour starvation groups.

With the spray containing 0.1 per cent. of pyrethrins, the dosages retained by males in the 4- and 6-hour starvation groups are likewise almost equal. By analogy with the flies exposed to the stronger spray, it would appear likely that, in these groups of male flies also, the rate of knock-down was close to its upper limit. As starvation time increased from 2 to 6 hours, the continuous declines in both the dosage on female flies and the mean time before knock-down are strong indications that the maximum rate of knock-down for females was not attained in the tests with the 0.1 per cent. spray. It is a well-established fact that female flies are less susceptible to the paralyzing action of pyrethrins and are knocked down less rapidly than male flies.

III. DISCUSSION

(a) The Effect of Pre-Test Starvation on Mortality of Flies treated with Pyrethrins

It has been shown that when houseflies were starved for 1 to 6 hours and then exposed immediately to pyrethrum sprays by the Peet-Grady method, mortalities were higher than those produced among flies which had been fed continuously before exposure. Furthermore, the mortalities caused by the sprays increased with pre-test starvation time.

The above statements are true also of flies which were given, individually, uniform dosages of pyrethrum solutions. However, the shapes of the probit mortality-starvation time curves, obtained by these two methods of treatment with pyrethrins, were found to be markedly dissimilar. The chief difference between the two methods was that the dosage applied to individual flies was constant, while the effective dosages received in the Peet-Grady tests were not controlled.

Colorimetric determinations of the amounts of spray retained by flies after exposure in Peet-Grady tests revealed that the mean dosages varied with starvation time.

(b) The Effect of Pre-Test Starvation on Factors which influence the Dosage received by Flies during Exposure to Pyrethrum Sprays

Evidence has been given that the rate of knock-down increased with starvation time. This increase is particularly noticeable in the starvation time range 0 to 2 hours with the 0.2 per cent. spray, and over the whole range 0 to 6 hours with the 0.1 per cent. spray. For both sprays, the percentage of unparalyzed flies in flight, and the speed of flight, increased with starvation time up to 2 hours, but thereafter appeared to remain constant. The reductions in flying time due to the greater rate of knock-down will tend to reduce the dosage received by starved flies relative to fed flies, while the increases in percentage of flies in flight and in speed of flight will have the opposite tendency.

For the starvation time range 0 to 2 hours, it cannot be determined from the available data alone whether the greater activity of the flies starved for 2 hours outweighs their higher rate of knock-down relative to flies continuously-fed or starved for 1 hour, and it can only be inferred from the data on dosage retained after exposure that it does so.

In the tests with the spray containing 0.1 per cent. of pyrethrins, activity appeared to be constant among flies starved for 2 to 4 hours, and the rate of knock-down increased considerably with starvation time. The lack of a precise method for measuring fly activity did not permit further experimentation to determine whether or not the dosage retained after exposure is proportional to the mean duration of flight when activity is constant. Furthermore, proportionality is uncertain because of the changing character of the spray mists during the flight periods, the possibility that pyrethrins may be preferentially absorbed from the dyed sprays, and because the amounts of insecticide retained after exposure may not be proportional to the total amounts collected. However, the evidence presented shows that the mean time before knock-down and the mean dosage retained after exposure decreased together as starvation time increased from 2 to 4 hours, and one could confidently predict that the mean flying time would also bear a similar relationship to dosage retained. Hence among the flies starved for 2 to 4 hours, the decrease in dosage retained after exposure which has been shown to accompany increase in starvation time was most probably due to the decrease in mean flying time. When starvation time was increased further, to 6 hours, the rate of knock-down also increased and the dosage retained by female flies decreased accordingly. But the dosage retained by males remained at the 4-hour starvation level. This is thought to indicate that, under the test conditions, the rate at which male flies were knocked down approached close to an upper limit in flies starved for 4 hours.

The results obtained in tests with the spray containing 0.2 per cent. of pyrethrins were, in general, similar to those with the weaker spray. However, very little change occurred in rate of knock-down when starvation time was increased from 2 to 6 hours. Obviously both male and female flies in these groups were paralyzed at rates which were close to maximal under the experimental conditions. Thus, of the two principal dosage-determining factors, fly activity appears to have been constant, while rate of knock-down approximated to constancy. Therefore, it is not surprising that the mean dosage retained by male flies in the 6-hour starvation group was almost identical with that retained by males starved for 4 hours, while for females, the mean dosage was virtually constant for the starvation time range 3 to 6 hours.

(c) *Comparison of the Effects of Pre-Test Starvation on Mortality of Flies in Peet-Grady and Uniform-Dosage Tests*

It has been shown in Figure 3 that when the dosage received by individual flies is constant there is a linear relationship between mortality, expressed in probits, and pre-test starvation time. It is fairly certain that a different uniform

dosage would result in a parallel line. Furthermore, if the technique of dosing could be modified so that, while the groups of flies at different starvation periods received the same mean dosage, the individual dosages varied within limits, and were distributed in each group either at random or adjusted to the ability of individual flies to resist paralysis, then it is also fairly certain that a parallel line would result. This latter condition of adjusted dosage would occur in Peet-Grady tests if the mean effective dosage received by the flies were constant for all starvation groups. In consequence, the uniform-dosage lines in Figure 3 may be superimposed with safety on the Peet-Grady curves in Figure 2, for convenience passing through common points at zero starvation time. These lines may then be taken as reference lines which represent the relationships of mortality to starvation time which would be obtained in Peet-Grady tests if the mean effective dosages received were constant for all starvation groups.

Superimpositions were made in the above manner and the differences between the ordinates to the curves and the uniform-dosage lines were determined. These differences, which, for convenience, have been termed "deviations," are shown plotted against starvation time in Figure 5.

Now at any starvation period, the mortality deviation reflects the difference between the effective dosages received by flies starved for this period and flies fed continuously. For comparative purposes, the differences between the mean dosages retained after exposure by flies in the various starvation groups and those retained by continuously-fed flies are also given in Figure 5, where, likewise, they have been termed "deviations." The correspondence between the two sets of data thus presented is most striking.

In both mortality and dosage deviations for male flies, there is an initial increase, with starvation time, followed by a decline. Furthermore, both deviations change sign in the vicinity of 4 hours of starvation time. The correspondence is not quite perfect, since the maximum deviation in mortality was shown by flies starved for 1 hour, while the maximum deviation in dosage occurred at 2 hours.

The mortality and dosage deviations for females show an even closer correspondence than those for males, for the maximum values occur at 2 hours of starvation time in both sets of data. Neither of the deviation "curves" for females intersect the lines for constant dosage, and there is a strong indication that both deviations become constant when starvation time exceeds about 4 hours. This flattening of the "curve" for dosage retained, when both fly activity and rate of knock-down reached maximum values, was discussed in Section III (*b*), and the corresponding flattening of the mortality deviation curve strongly suggests that, under these conditions in Peet-Grady tests, the only remaining variable was the susceptibility of the flies to pyrethrum poisoning, as in the uniform-dosage tests.

Taken as a whole, the correspondence between the mortality and dosage deviations is good and leaves little room for doubt that the influence of pre-test starvation on the effective dosages received in Peet-Grady tests is the primary factor responsible for the dissimilarity between the two methods of treatment with pyrethrins, in the relationship of mortality to starvation time. It also lends

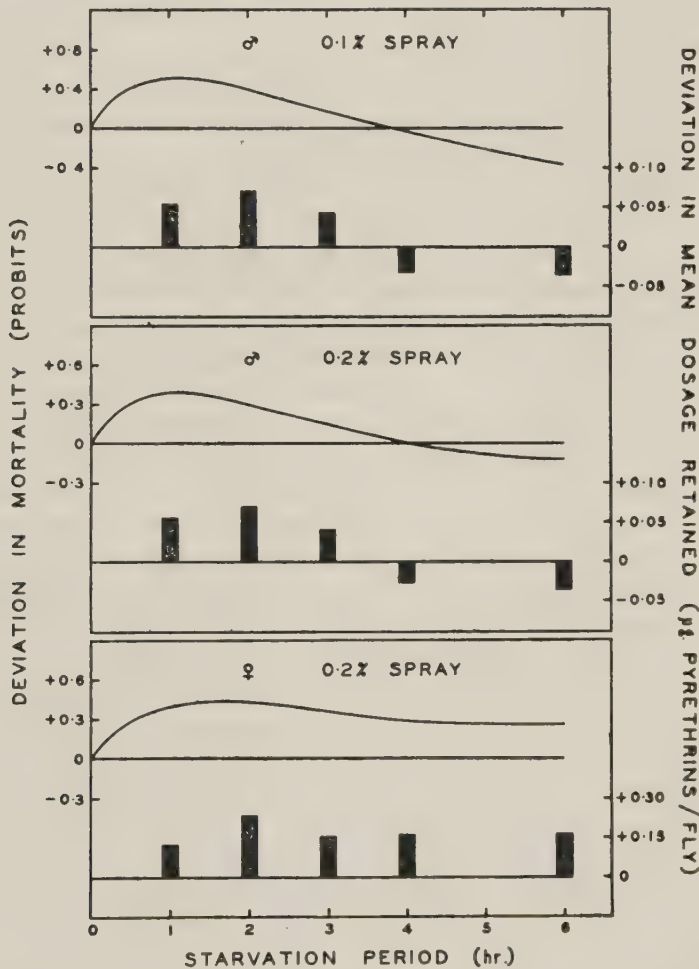


Fig. 5.—Deviations in mortality and mean dosage retained after exposure plotted against starvation time.

strong support to the statement made earlier that the toxic effect of the changing dosage to which an individual fly is subjected before and after knock-down in Peet-Grady tests is probably not greatly different from the effect of a constant dosage equal to the dosage retained after exposure.

(d) General

The description of the official Peet-Grady method as given in Soap Blue Book (1943) states somewhat ambiguously that "On the day of testing the flies should be fed at least 3 hr. before spraying begins." The procedure for feeding the test flies is more rigorously defined in the 1946 revision which states that "satisfactory food must be available to the flies at all times." This is a definite improvement, but there still remains the possibility that some of the flies may be disturbed from feeding during the one or two hours immediately prior to testing.

It has been observed that when flies are disturbed from the food pads by such actions as moving the cage or merely by moving objects rapidly past the cage, they fly in an agitated manner for a few seconds, and eventually most of them alight on the top and sides of the cage. A considerable time, usually exceeding 40 minutes at 27°C. in flies which have had continuous access to food, elapses before normality in the proportion of flies feeding is regained.

A single disturbance before testing, such as might occur when a cage of flies was being transferred from the rearing room to the testing room, may not be of great importance, but if flies for each test in a series are drawn from a single stock cage, as described in the Small-Group procedure of the Peet-Grady method, they will be disturbed every time a sample is taken. In this procedure the number of tests in a series is often as great as 10, and, since each test is completed in not less than 15 minutes, it is highly probable that some of the flies removed from the stock cage in the last few sample batches will have been starved for a considerable time. Even if two testing chambers are in operation simultaneously, it is doubtful whether 10 tests could be accomplished in less than 2 hours, during which a disturbance of the flies occurs at intervals shorter than the time taken for them to return completely to their normal fully-fed state.

The alternative method of sampling in the Small-Group procedure is as follows: "Samples may be taken also by discarding the first 100 flies and then counting 50 flies into each of a series of small cages. 100 flies are counted into the last cage and then, starting with the next to last cage and working backwards, 50 flies are added to each. Flies remaining in the stock cage are discarded." Thus all groups of test flies have to be removed from the stock cage before the first test is begun. If the flies are not fed after sampling, those in the last few groups will be starved for considerable periods before they are tested. If food is supplied to the sampled flies, those in the first few groups may not have sufficient time before testing to return to their fully-fed condition.

Thus in the Small-Group procedure the groups of flies taken from a stock cage will vary in the amount of starvation which is caused unavoidably by the disturbance of sampling, or deliberately by not feeding the flies after sampling.

This investigation has shown that short periods of pre-test starvation result in considerable increases in mortality when flies are sprayed with pyrethrins. It is therefore suggested that better uniformity between groups of test flies could

be obtained by drawing all samples of flies from a stock cage at least 2 hours before testing and ensuring that food was available, and the flies not disturbed, from sampling to testing time. Such a procedure would increase the chances of all flies being well fed at the time of testing.

In the Large-Group procedure each test group is fed as a separate unit, and providing the flies are not disturbed, the groups should be equally well fed at the time of liberation for test.

Although sampling procedure and the treatment which flies receive before testing have been discussed specifically for the Peet-Grady method, it is clear that whatever method for testing fly sprays is used, special attention should be given to the sampling technique and the pre-test handling of flies, to ensure that starvation, caused by such disturbances, does not introduce unnecessary variability between test samples.

Among houseflies reared by the Peet-Grady method, there is considerable variability between cultures in the susceptibility of the flies to pyrethrins. To conform with Peet-Grady standards, the strain of flies used must be of such susceptibility that the Official Test Insecticide, containing 0.1 per cent. w/v of total pyrethrins, will cause a mortality between 30 and 55 per cent. Thus in the framing of the method this variability has been taken into account, and a liberal range of susceptibility has been allowed. Nevertheless, the selection and maintenance of a suitably susceptible strain may be difficult and time-consuming.

If the strain of flies becomes too susceptible to pyrethrum sprays, it is doubtful whether any remedial measures could be applied to it, except perhaps the rearing of succeeding generations from those flies which survived exposure to the sprays. But if the strain is too resistant, the present investigation suggests a simple method of preconditioning the test flies in order to increase the mortality caused by the Official Test Insecticide.

It was shown in Figure 1 that the concentration-mortality regression lines for flies starved for 2 hours and flies fed continuously are parallel. Thus an increment of pyrethrum concentration produces equal increments of mortality among flies starved for 2 hours and those which are fed continuously before testing. It is therefore permissible to use flies which have been starved for 2 hours for the evaluation of fly sprays containing pyrethrins.

In 10 Peet-Grady tests, the 0.1 per cent. pyrethrum spray produced a mean mortality of 27.6 per cent. among flies starved for 2 hours as compared with 10.8 per cent. in continuously-fed flies, a difference of 16.8 per cent. Hence flies, for Peet-Grady tests, which are of such susceptibility that the Official Test Insecticide kills only, say, 15 per cent. when they are tested in the fully-fed condition, need not be discarded as being too resistant, for at least 30 per cent. of them will be killed by the spray if they are preconditioned by 2 hours of starvation immediately prior to testing.

Although confirming tests were not performed, it is likely that the regression lines for flies starved for periods slightly shorter or longer than 2 hours would also be parallel to the line for fully-fed flies. If this is so, it should be possible to vary, within certain limits, the level of mortality produced by the standard spray simply by varying the time for which the test flies are starved before exposure, the limiting factors being the susceptibility of the fully-fed flies and the maximum starvation time tolerated without loss of vigour.

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V. REFERENCES

- ANON. (1943).—Peet-Grady Method. *Soap Sanit. Chem.*, Blue Book.
——— (1946).—*Ibid.*
DAVID, W. A. L. (1945).—Insecticidal sprays and flying insects, *Nature* 155: 204.

ENERGY TRANSACTIONS IN THE SHEEP

I. THE BASAL HEAT PRODUCTION AND HEAT INCREMENT

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Summary

In this paper existing knowledge of the respiratory metabolism of the sheep is summarized briefly, the outstanding problems are revealed and the first of a series of experimental investigations of the physiology of energy utilization by the sheep is reported.

The main experiment comprised a series of critical determinations of the complete energy balance sheets of seven mature Merino ewes, each observed at five levels of food intake which extended from approximately $\frac{1}{2}$ maintenance to 2 maintenance.

The respiration calorimeters employed are described and methods of computing the heat production of the ruminant from its respiratory exchange are discussed. The parameter $W^{0.73}$ was used to reduce to a common basis the observed heat productions of animals of different weights.

The fasting (basal) heat production, observed at all levels of intake under critical basal conditions over the 24-hr. period beginning 72 hr. after the previous feed, revealed that a post-absorptive state had not been reached in this interval in the case of the animals fasted from the highest (2 maintenance) level of feeding. The mean under these conditions was 74.5 kg. cal./ $W^{0.73}/24$ hr. The fasting heat productions of the animals previously subjected for 10 weeks to the semi-starvation ($\frac{1}{2}$ maintenance) level indicated an inanition effect. The mean under these conditions was 59 kg. cal./ $W^{0.73}/24$ hr. The heat production 72-96 hr. after feeding at all other levels varied but little from a mean of 68 kg. cal./ $W^{0.73}/24$ hr., and it is suggested that this is a critical estimate of the basal (fasting) metabolism of the Merino sheep.

Above the maintenance level, the heat productions of the fed animals were related linearly to the intakes of available energy when reduced by the parameter $W^{0.73}$, and the heat increment of the fodder was derived directly from this regression.

Extrapolation of this regression to the heat production axis indicated the "true basal" heat production, β , to be 54 kg. cal./ $W^{0.73}/24$ hr. In a discussion of the significance of β it is shown that the well-observed data of Forbes *et al.* (1928, 1930), from experiments with steers, when treated in this way, yielded a value for β , which is not significantly different from that derived in this experiment, the value being 54.56 ± 2.30 kg. cal./ $W^{0.73}/24$ hr. for the sheep and 51.85 ± 3.38 kg. cal./ $W^{0.73}/24$ hr. for the steer. The "true basal" requirements of energy of the steer and sheep are thus very closely related to the 0.73 power of their body weights. It is argued that β is the "true basal" requirement of energy and that B , the observed basal (fasting) heat production, is β , plus the heat increment of the tissue substance drawn upon to provide this energy.

The heat increment of the source of energy drawn from the tissues during fasting was estimated in this way to be 20 per cent.

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From these figures the heat production at submaintenance levels of energy intake was shown to be the "true basal" energy intake, β , increased by the heat increment of the fodder plus the heat increment of the energy source from the tissues drawn upon to make up the energy deficit.

The physiological bases of Kellner's Starch Equivalence System and of Armsby's Net Energy Principle are discussed in the light of these findings.

In conclusion, the observed high heat increment of the fodder is discussed in its relationship to Rubner's theory, and it is suggested that the very high specific dynamic effect of carbohydrates when fed to ruminants is due to the high thermodynamic costs involved in the introduction, into useful channels of metabolism, of the fatty acids which result from fermentation in the paunch.

The combustible energy of ration 541, which consisted of 5 parts of grain (wheat), 4 parts of lucerne meal and 1 part of cane molasses, was 4.5 kg. cal./g. dry weight. The energy available from this was 70 per cent. (3.15 kg. cal./g.), and as the heat increment which supervened on feeding it was 38 per cent., the net (useful) energy was 1.95 kg. cal./g. dry weight. As β , the "useful" energy requirement of the sheep was 54 kg. cal./ $W^{0.73}/24$ hr., the quantity of this diet necessary to maintain a sheep at rest in an environmental temperature within the zone of its thermic neutrality is 28 g./ $W^{0.73}/24$ hr.

I. INTRODUCTION

Lavoisier's observation that his colleague Seguin consumed more oxygen after eating a meal (Seguin and Lavoisier 1789) revealed a fundamental physiological problem which has not been satisfactorily solved. A little over half a century after this, the discovery that carbon dioxide production increases under similar circumstances (Scharling 1843) rendered more simple the experimental investigation of the phenomenon, and then only a few years elapsed before both the basal metabolism and the increase in heat production which supervenes on feeding were defined. Edward Smith, after observing the rate of elimination of carbon dioxide by human subjects under a variety of conditions, concluded that "... there is a normal (basal) level below which the system does not pass in health and wakefulness and which is tolerably uniform ... in complete abstinence from food ..." and he expressed the view that "... foodstuffs may fitly be divided into two classes, viz. those which excite certain respiratory changes and those which do not." (Smith 1859*a*, 1859*b*.)

A great deal of experimental effort during the latter half of the 19th century and up to the present day has been devoted to the task of explaining the origin of these respiratory changes which reflect the increase in heat production that supervenes in all animals after food has been ingested, but no adequate theory of the mechanisms responsible has been forthcoming. Neither the *Verdauungsarbeit* hypothesis (von Mering and Zuntz 1877) nor the plethora theory (Lusk 1928) affords a satisfactory explanation, and the extensive literature based on Rubner's concept of specific dynamic action provides, in its present form, an analytical description of the phenomenon rather than a complete explanation of the underlying mechanisms.

Although the experiments described here bear directly upon this problem, they were undertaken to meet a purely practical end. The provision of rations

for the maintenance of flocks during the periodic droughts experienced in the pastoral areas of Australia entails long and expensive haulage, and so it is essential for economic reasons that rations which might serve this purpose should be as concentrated as possible. With this in view a comprehensive study was made of a mixed fodder which would supply in relatively small bulk the materials essential for the complete nutrition of the sheep. The investigation led to the determination of the extra heat production which supervenes when the ration is fed, for obviously the energy dissipated in this way must be considered a debit in the matter and energy balance sheet when an assessment is made of the capacity of the fodder to provide for the animal the energy necessary to support its living processes.

The fact that foodstuffs differ widely in their ability to induce extra heat production in the animal organism was recognized by physiologists early in the history of this subject, but it was overlooked in the first attempts to evaluate the capacity of a fodder to provide energy to farm animals. Kellner, who in his earlier writings* failed to appreciate that heat production is not an end but an incident of metabolism, assumed the function of the maintenance ration to be the provision of fuel for the support of body temperature and so concluded that the fodder would have two distinct values, its capacity to provide the energy for maintenance and its capacity to provide the energy for production of body substance (Kellner 1905). Armsby, however, dismissed the conception (Kellner and Kohler 1900) which led to this conclusion by demonstrating unequivocally that only 60 per cent. of the metabolizable energy† of a sub-maintenance ration actually contributed to the quota of energy necessary to sustain life in an ox; the remainder merely increased the heat production which was already sufficient to maintain the animal's body temperature (Armsby and Fries 1903).

The quota of the available energy that is dissipated as heat without apparently serving any useful purpose in metabolism‡ is, however, still considered to vary with the level of intake (cf. Forbes and Voris 1943). This conclusion is not convincing. It is based on an apparent difference in the relationships between food intake and heat output above and below the maintenance level. The metabolic phenomena at submaintenance levels are complicated, and the means of computing the heat increments under these conditions are inadequate if fasting metabolism is used as a base. When the available energy of the fodder is insufficient to provide for the energy requirements of the animal,

*This was corrected in the later editions of Kellner's "Die Ernährung der landwirtschaftlichen Nutztiere."

†The "metabolizable energy" is the combustible energy of the fodder minus the sum of the combustible energy of the visible excreta and of the methane. This term is synonymous with "available energy."

‡i.e. the "heat increment"

the "heat increment" fraction of the total heat that is dissipated is made up of two variables, viz. the heat increment of the fodder and the heat increment of the tissue substance drawn upon to make up the net energy deficit.*

Evidence is submitted in this paper to prove that the heat increment of the fodder does not alter over the usual range of feeding, and to show that, for any diet, the quota of the available energy which is useful to the animal is unchanged by the level of feeding, even at submaintenance levels, provided that the animal is in an environmental temperature within the zone of its thermal neutrality.

Complete balance sheets of the energy transactions in seven sheep were determined under critical conditions at five levels of intake in which the available energy ranged from approximately half that required for maintenance to approximately twice maintenance. The fodder, when it was fed at maintenance level, provided in ample quantity all the nutritional requirements of the animals. From the data obtained, the relationships between the available energy and the heat production of each individual could be determined with considerable precision.

II. THE EXPERIMENT

(a) *The Experimental Regime*

The experimental animals were fed at each level for a period of 10 weeks, the collections from which the balance data were derived being made during the 14 days of the 9th and 10th weeks of this cycle. Immediately before and after each collection period the animal spent two periods each of 2 days in the respiration chamber of the calorimeter. When the balance and heat production estimations were completed, food was withheld for 3 days and the heat production during fasting was determined over the 24 hr. of the 4th day of fast. Food was then provided, the ration being altered to the next level and the cycle repeated. During each pre-period the animals had ample time to establish a steady nutritional state. Wool growth was estimated by shaving patches (15 cm. by 10 cm.) delineated by tattoo lines on either shoulder. The relationship between this and the nutritional plane will be discussed in another publication.

Of the 8 animals treated in this way, 4 were fed at ascending and 4 at descending planes of nutrition, the range of intakes extending in stages from $\frac{1}{2}$ maintenance, through maintenance and $1\frac{1}{2}$ maintenance to 2 maintenance. After

*H. P. Armsby's term "net energy" was defined by him as "the metabolizable energy minus the expenditure of energy by the body which results from the ingestion of food" (Armsby 1917, pp. 279-80). Forbes's more comprehensive definition is: "The total of the expenses and losses of food utilization in terms of energy subtracted from the gross energy of the food yields the net energy available for maintenance and production, and these expenses and losses are (1) the potential energy of the visible excreta and of the methane produced by carbohydrate fermentation, and (2) the heat increment—this latter comprising not only all direct expenditures of energy in prehension, mastication, deglutition, fermentation, rumination, peristalsis, digestion, transportation, anabolism, dynamic stimulation and excretion, but also of any direct increase in the heat production either through voluntary or involuntary activity which has resulted from the consumption of food." (Forbes *et al.* 1928).

this programme was completed the nutritional plane of some was raised and that of others lowered to levels within the range between maintenance and 2 maintenance to provide further data. The same pre-period to establish a stable state was adopted with these. Subsequently the experimental animals were employed in a study of the influence of the nutritional status on the fasting metabolism.

(b) *The Fodder, 541*

The components of the fodder were 5 parts of crushed grain (wheat), 4 parts of finely chaffed, good quality lucerne hay, and 1 part of cane molasses. These were heated with steam, intimately mixed, and then compressed into cubes (approx. 2 cm.³) by means of machinery used to prepare concentrates employed by the pastoral industry for the supplementary feeding of sheep. The finished cubes were then dried and stored in iron bins.*

The fodder in this form was easily handled and readily sampled. It was evidently very palatable as the animals avidly sought it and consumed within 2-3 hr. the whole of the rations offered each day.

As care was taken to mix the large batch of material from which the cubes were made for this experiment, the composition was reasonably constant. However, during the collection periods, samples were drawn daily from each ration offered and it is from analyses of these samples that the nitrogen and gross energy intake figures discussed elsewhere were derived. Analyses of a composite sample drawn from the whole batch yielded the following figures: crude protein (N x 6.25) 14.4 per cent., crude fat (ether extractive) 1.7 per cent., crude fibre 8.7 per cent., and ash 4.75 per cent. The material contained 7.3 per cent. of cellulose, 3.9 per cent. of lignin, and 43 per cent. of starch. The "total fermentable reducing substances" estimated as glucose after acid hydrolysis was 53 per cent., the free glucose 0.9 per cent., and sucrose 5.8 per cent. The concentration of carotene was 7.8 mg./kg. The major ash constituents were: calcium 0.54 per cent., phosphorus 0.36 per cent., sodium 0.033 per cent., potassium 1.39 per cent.; and among the minor constituents were: copper 9 µg./g., and cobalt 3 µg./g.

Common salt and some extra calcium were provided in addition, and once a week 15 ml. of cod liver oil was administered to each animal. These adjuncts were omitted during the periods when the excreta were collected.

(c) *The Experimental Animals*

Eight mature, strong-wool Merino ewes (Anama strain), aged approximately 3½ years at the beginning of the experiment, were employed. These animals which were of equable temperament were trained for a period to accustom them to the environment of the metabolism cages (Marston 1935) from

*We are indebted to Messrs. Thorpes Ltd., Sydney, who prepared this material at our direction and presented it to the Division for this experiment.

which, during the 15 months of the experiment, they were absent only for the occasional brief periods which they spent in the respiration calorimeters. Other than one which sickened and was removed early in the experiment they remained healthy and vigorous during the whole term. They accepted the conditions and when they had become accustomed to the cages they spent most of the day reclining in their natural posture of repose. They remained alert, however, and although they became very tame with the attendants, intrusion of a stranger into the annexe in which they were housed would cause all to stand and to show their displeasure by adopting an attitude of defence. They were fed at noon, and it was their habit to consume the rations within 2-3 hr. They showed no dislike of the conditions in the respiration chamber and behaved as if they were in the metabolism cages to which they had become accustomed. It is evident that sheep of this strain are temperamentally suited to the critical experimental conditions demanded by energy balance studies, for they clearly feel secure in close quarters and readily accept the exacting regime required for successful experiments.

(d) Collection of Urine and Faeces

At the beginning and end of each collection period the animal was induced to void its urine by the simple and rarely failing device of holding its nose for a few seconds so as to render breathing more difficult. This treatment almost invariably leads the animal to micturate when released. Although a catheter may be passed with ease in the ewe, this means of draining the bladder was employed only on rare occasions, and then with strict aseptic precautions to preclude infection.

The 24-hr. samples of urine were combined and preservatized. At the end of the 14-day collection periods the total volume was measured and aliquots drawn for chemical analysis, etc.

The faeces were dried each day in an apparatus designed to prevent the loss of volatile nitrogenous constituents.*

The total dry weight of the faeces passed during the period was determined and the samples for chemical analysis and combustible energy determinations were drawn and reduced to 100 mesh in a C. and N. Mill. At each stage samples were withdrawn for moisture checks, all analyses being referred to constant weight at 105°C.

(e) The Determination of the Combustible Energy and Nitrogen Content of Excreta

The combustible energy of the faeces and of the urine was determined with an Emerson oxygen bomb calorimeter fitted with an adiabatic jacket. In the preparation of samples for the estimation, accurately weighed pellets (approx. 2 g. dry wt.) of the dried, finely ground faeces were compressed around the fuse

*This apparatus will be described in detail elsewhere.

wire by means of a die; the urine (20-50 ml. depending on the dilution) was dripped slowly into a light platinum capsule, 2.5 cm. in diameter, and evaporated to dryness in a stream of hot dry air, the rate of delivery into the capsule being arranged so that only sufficient urine to cover the bottom of the capsule was allowed to accumulate. The samples were fired electrically in oxygen at 300 lb./sq. in. In some cases when complete oxidation of the urine was difficult to achieve, a weighed quantity (1 g.) of pure sucrose was added to the capsule before the urine was admitted and the heat of combustion of the added sucrose was deducted later from the observed calorific value of the mixture.

Nitrogen was determined in the urine and faeces by the Kjeldahl method, heating of the digest being continued for 8 hr. after the mixture had cleared.

Three samples of each were assayed for combustible energy and nitrogen, and the results were within ± 0.5 per cent.

(f) *The Respiration Calorimeters*

Two open-circuit respiration calorimeters of practically identical design were employed for the estimation of heat production of the sheep. Since Lines (1938) described the first of these, several modifications of the mechanical parts have been introduced to ensure greater reliability, and a number of attachments have been added to increase the scope and convenience of the apparatus.

The general arrangement is indicated in Figure 1. The stall occupied by the animal is very similar to that of the metabolism cage (Marston 1935), and the arrangement of the food and water troughs and the means for separating and collecting the faeces and urine are the same. In consequence, an animal which has become accustomed to the metabolism cage is not disturbed by the environment of the calorimeter. A second ration is held in a hopper, from which it may be released into the feeding trough at will without opening the chamber.

When lowered, the cover of the chamber rests on a sponge rubber gasket immersed in a water trough to ensure a complete seal. It then encloses an air space of approximately 2 cubic metres. The atmospheric conditions within the chamber are controlled by circulating the air through a conditioning device described by Lines (*loc. cit.*) and by electric heaters operated by a Minimax thermograph and regulator. The former serves mainly to reduce humidity—it is not of sufficient capacity to lower the air temperature more than a degree or so when the external temperature is high. To minimize radiation the whole of the exterior of the chamber, excluding the plateglass window at the top through which the interior is illuminated, is insulated with a thick layer of felt covered with painted canvas.

A device which records the number of times the animal rises from its natural posture of repose and the duration of the periods spent in standing has been added. The path of a light beam which falls on an emission-type photocell is interrupted when the animal is standing, and an electronically operated mechanism registers by an impulse meter the number of interruptions of the beam and an electric clock records the duration of these interruptions.

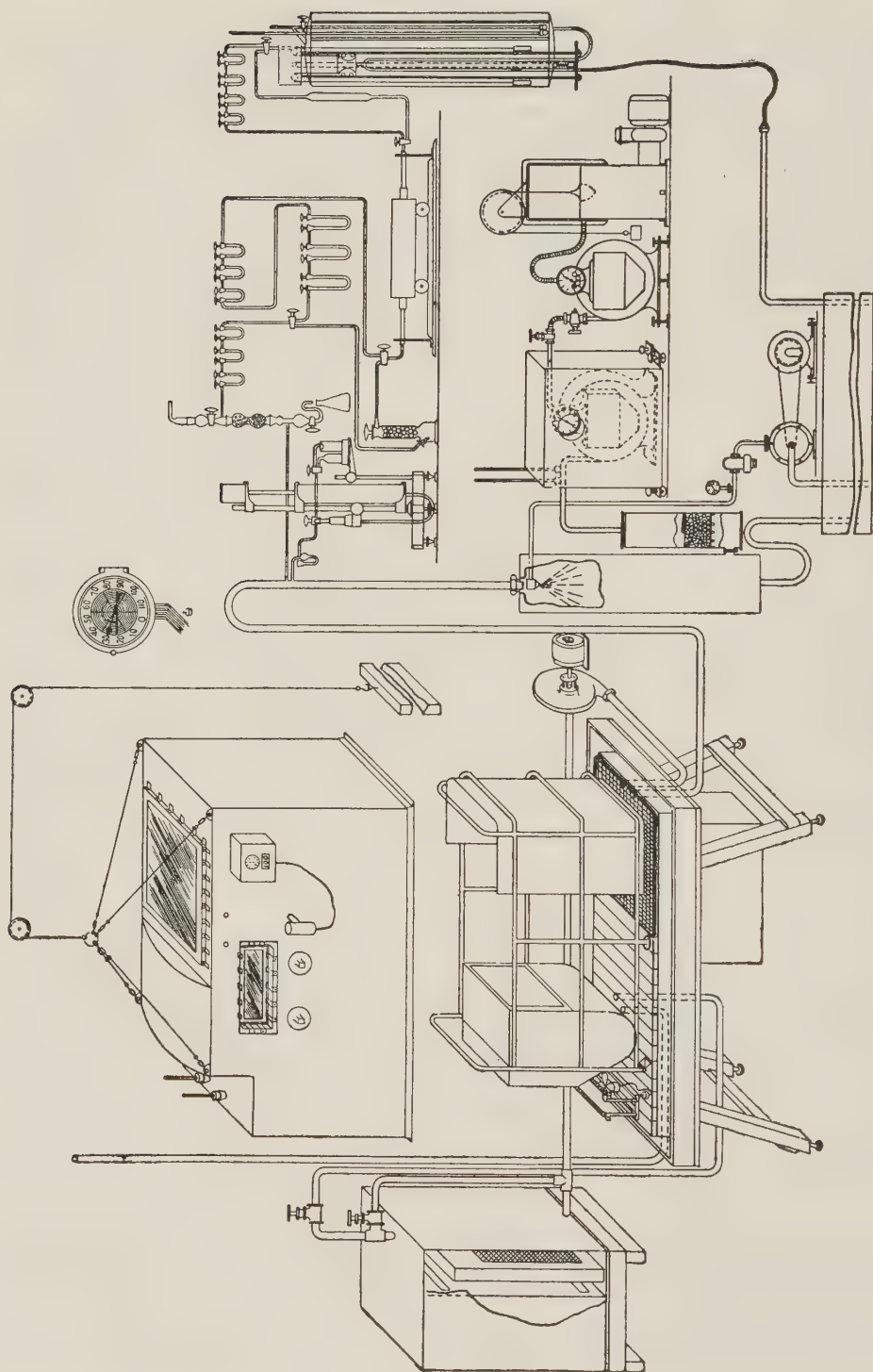


Fig. 1 (For legend see opposite page)

The stream of air which ventilates the chamber is taken from outside the annexe in which the calorimeter is housed. It is thoroughly mixed in the chamber by passing through the conditioning circuit. The effluent stream is drawn through a spray-tower where it is saturated with water vapour and brought to an even temperature. Thence it passes through two standardized wet-type gas meters, the first of which is protected against heat exchange by an insulated box and is used as the volume recorder; the second acts as a check to indicate any gross fault in the operation of the first. The rate of ventilation (between 1,200 and 1,500 l./hr), which is kept constant with a flow regulator, is arranged so that the increase of CO_2 partial pressure within the chamber never exceeds 1 per cent., at which level respiration of the animal is not stimulated. The temperatures at which the estimations are made are not allowed to fall below 18°C . nor to exceed 32°C .—within this range of temperature and of the humidities which prevail in the chamber the animal is able to regulate its body temperature without any stimulation of its heat production.

Samples of the effluent air stream are drawn over mercury at a constant speed with a sampling device operated by a synchronous motor. As the rate of flow of the stream from which it is drawn is constant, the sample represents a true aliquot. Bailey bottles (Bailey 1921) are used to transfer the samples to the volumetric gas analysis apparatus.

The overall efficiency of the apparatus is tested periodically by burning alcohol in the chamber. The Mariotte-bottle lamps used for this purpose are regulated to burn the fuel so that the O_2 consumption and CO_2 production will

Fig. 1.—The main features of the respiration calorimeters used for the determination of the heat output of the animals may be gathered from this diagram of one of them. The pen is hinged in front to allow access to the apparatus for separation of urine and faeces. The food bin is removable; it slides on the top rails and so affords a means of altering the space within the pen. The water trough attached to the off-side of the cover is not shown. The heating elements and fan indicated beneath the food bin comprise a unit which may be removed at will to allow access to the pen. The cover of the chamber is fitted with subsidiary gear for recording and regulating the air temperature within the chamber; for indicating the movements of the animal; and for inspection of the animal and introduction of tubes etc., when the animal is enclosed in the chamber.

The devices for sampling, metering, etc., are indicated in elevation. The effluent air stream is saturated with water vapour and brought to constant temperature in the spray tower and then measured in the two wet gas meters. The rate of ventilation is kept constant by the regulating device shown between the meters and the air pump.

The aliquot sample (300 ml.) for the volumetric determination of CO_2 increase and O_2 deficit is drawn at constant speed over mercury by the apparatus shown. This sample is freed from water vapour before collection. The sample for the gravimetric estimation of CO_2 and CH_4 is drawn at constant speed by a large tonometer (100 l.). This sample is first freed quantitatively from H_2O and then passed into a chain of U-tubes in which the CO_2 is absorbed. It then passes through a silica tube packed with cupric oxide maintained at 720°C . in an electric furnace, and thence to another series of U-tubes in which the H_2O and CO_2 arising from the combustion of the inflammable gases are absorbed. The mechanical arrangements are robust in design so that they may be run continuously for periods up to a fortnight or more.

be approximately that of the sheep. Recoveries of between 98.5 and 100 per cent. are obtained. The lower figures are mainly due to incomplete combustion, as CO_2 and H_2O arising from unburned alcohol are recovered under these circumstances in the absorbers of the methane combustion chain.

(g) *The Gas Analyses*

The aliquot sample of the effluent stream from the chamber is analysed for CO_2 and O_2 in the modified Carpenter-Haldane apparatus (Carpenter, Fox, and Sereque 1929). In the observations reported in this paper three analyses of each sample were made immediately subsequent to a critical analysis of the outside air. This practice is adopted to ensure that the apparatus is in satisfactory working order before proceeding. The CO_2 concentration was estimated to ± 0.003 per cent., and the O_2 to ± 0.005 per cent. The experimental error arising from gas analyses was thus considerably less than ± 1 per cent. The volumetric estimate of CO_2 concentration was checked by gravimetric analysis.

(h) *The Gravimetric Estimation of Methane and of Carbon Dioxide*

The CH_4 and CO_2 evolved by the animal are estimated in a large integral aliquot (50-75 l./24 hr.) drawn from the effluent air stream by means of a calibrated tonometer in which the water level is lowered at a constant speed with an overflow mechanism operated by a synchronous clock motor. As the tonometer works at a slightly negative pressure, the extent of which depends on the closeness of the packing of the adsorbents, the observed volume is corrected from the water barometer prior to reducing to N.T.P. and correcting for water vapour pressure. The total volume of dry gas at N.T.P. drawn from the ventilation stream is derived from this by adding to it the sum of the volume of the CO_2 absorbed plus three times the volume of the CH_4 , as 2 vol. O_2 are removed together with 1 vol. CH_4 when the products of the complete combustion of CH_4 are absorbed. The rate at which the effluent stream is drawn through the absorption and combustion chain is between 2 and 3 l./hr., and this is well within the tolerance for complete oxidation and quantitative absorption.

After complete removal of the H_2O by H_2SO_4 and anhydrous magnesium perchlorate (anhydrite) the CO_2 is absorbed in soda-asbestos (ascarite 100) in a chain of large U-tubes, guarded on either side by tubes containing anhydrite. The CO_2 content of the effluent air is computed from the gain in weight of the CO_2 absorbers and the corrected volume drawn by the tonometer.

The CH_4 is estimated by passing the H_2O - and CO_2 -free effluent stream through a silica tube packed with copper oxide maintained at 720°C . (cf. Lugg 1938) and thence through a chain of small U-tubes packed, in order, with anhydrite, P_2O_5 , soda-asbestos (2), and P_2O_5 . The increases in the H_2O and CO_2 absorbers are corrected for H_2O and CO_2 arising from the small quantities of inflammable gases normally present in air which, according to Lugg, are approximately 4 p.p.m. H_2 and 2 p.p.m. CH_4 . Within the small limits of the experimental errors of the experiments under discussion the ratio of hydrogen to

carbon in the inflammable gases as inferred from the H_2O and CO_2 produced by combustion was 4 : 1, and there was no doubt that CH_4 was the only inflammable gas produced by the animals. There was, however, ample evidence of free hydrogen in the effluent stream from one animal which sickened and was removed from the experiment.

(i) *The Computation of the Heat Production from the Respiratory Exchange*

The heat production of the fed sheep was estimated from the observed O_2 deficit and the increased CO_2 and CH_4 concentrations in the air stream emerging from the respiration chamber, and the nitrogen in the urine, by a method based essentially on the procedure of Zuntz and Schumburg (1901) but modified to account for the combustible energy of the CH_4 arising from the fermentation of carbohydrate in the alimentary canal of the animal.

The determination of the heat produced by the ruminant entails special problems, as the greater proportion of the available energy from the carbohydrates of the fodder is derived from fatty acids produced by the fermentative activity of the rumen microflora. The chemical changes which result from fermentation in the rumen are exothermic (Marston 1948) and so it is not feasible to separate the heat produced by the animal itself from that produced by the micro-organisms concerned with the fermentations in its alimentary canal. Nor, on account of the fact that CH_4 , which is formed from the fermentation of carbohydrate, is evolved, can the overall heat production of the animal and its flora be determined from the respiratory exchange by methods that have been established and amply proven to be applicable to the determination of the heat production of animals with simple stomachs in which the production of inflammable gases is negligible. The latter difficulty may be overcome by computing the total heat production which would result if the CH_4 were completely oxidized along with the other products of rumen fermentation and subtracting from this total the heat of combustion of the CH_4 . To achieve this end, Andersen (1920, 1922) suggested that the O_2 deficit be increased by the O_2 (2 vol.) necessary to oxidize completely the CH_4 , and that the CO_2 evolved be increased by the CO_2 (1 vol.) which would result from this oxidation, and that the R.Q. and O_2 deficit derived from these augmented figures be used to compute the total heat that would be produced if the whole of the carbohydrate involved in the metabolic transactions of the animal and its flora were burnt. The actual heat output may then be determined by subtracting from this total the heat which would result if the CH_4 were oxidized.

The heat output computed in this way is a thermodynamically valid estimate, but it should be realized that it is a measure of the overall heat output, i.e. *the sum of the heat produced as an end result of the metabolic processes of the animal organism and the heat evolved by the fermentative activities of the symbiotic microflora of its alimentary canal*; and there is no apparent, direct means of separating these two factors. An assessment of the "free energy" of the

anaerobic dissimilation of cellulose by these microorganisms, computed from the combustible energy and carbon balance sheets of *in vitro* fermentations, suggests that the heat produced by the activity of the microflora in the rumen is in the vicinity of 6 per cent. of the combustible energy of the carbohydrate fermented (Marston 1948). This energy would be an integral part of the heat increment of the fodder as it is valueless in the economy of the animal other than when the environment is such that the animal dissipates more heat than is produced in the normal course of its metabolism.

The heat arising from the katabolic oxidation of protein was computed from the urinary nitrogen, and for this purpose Loewy's (1911) estimate that the katabolic oxidation of protein would produce 4.463 kg. cal./l. O₂ or 26.5 kg. cal./g. N (Lusk 1928) was adopted in preference to the estimate of Magnus-Levy (1907) which is 4.578 kg. cal./l. O₂ or 27.1 kg. cal./g. N, or to that derived by Mollgaard (1929) which is 4.639 kg. cal./l. O₂ or 28.1 kg. cal./g. N. Loewy's figures were computed from careful analyses of muscle protein and may possibly be low when the heat arising from the oxidation of vegetable proteins is considered, but in the assessment of the total heat produced by the fed animal these differences are not cogent. If protein katabolism were ignored altogether and the heat production were derived directly from the observed R.Q. and O₂ deficit by means of equation (3) (*vide infra*), the error involved in the computed heat production of any animal reported in this paper would not exceed 1.4 per cent.* and so the error which would result if any of these other estimates of the heat of combustion of protein were substituted for those of Loewy would amount to only a small fraction of 1 per cent.

The actual procedure employed in computing the heat output of the fed animals is as follows:

The corrected non-protein respiratory quotients (*R*) reported in Table 4 were derived from the expression:

$$\frac{\text{CO}_2 + \text{CH}_4 - 4.76\text{N}}{\text{O}_2 + 2\text{CH}_4 - 5.94\text{N}} = R \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where CO₂ = l. CO₂ eliminated/24 hr.

O₂ = l. O₂ deficit/24 hr.

CH₄ = l. CH₄ eliminated/24 hr.

N = g. N. in urine/24 hr.;

the factors 4.76 and 5.94 being respectively the CO₂ arising from, and the O₂ consumed in, the oxidation of an amount of protein equivalent to 1 g. urinary N.

*The R.Q. of katabolized protein is 0.802 and the heat evolved is 4.463 kg. cal./l. O₂ (Loewy 1911). The heat evolved by a mixture of fat and carbohydrate which would have the same R.Q. (0.802) is 4.803 kg. cal./l. O₂ (Zuntz and Schumburg 1901). As the greatest contribution that protein katabolism made to the total heat output of the animals at any nutritional level reported here was <20 per cent., the error involved by disregarding protein katabolism and computing from equation (3) would be 7 per cent. of this, i.e. <1.4 per cent.

The heat production (M) of the fed animal (plus that produced by its symbiotic microflora) was derived from the expression:

$$(\text{O}_2 + 2\text{CH}_4 - 5.94\text{N}) (4.686 + 1.23 \overline{\text{R.Q.}} - 0.707) - 9.44\text{CH}_4 + 26.5\text{N} = M \text{ kg. cal.} \quad (2)$$

where the constant 1.23 is the difference between the heat evolved per l. O_2 in the oxidation of carbohydrate (5.047 kg. cal.) and the heat evolved per l. O_2 in the oxidation of fat (4.686 kg. cal.) divided by the difference between the R.Q. of fat (0.707) and the R.Q. of carbohydrate (1.00). The heat of combustion of $\text{CH}_4 = 9.44$ kg. cal./l. and the heat evolved when protein is oxidized in katabolism is 26.5 kg. cal./g. N.

The simplified equation (3) was used for the determination of heat production when the animal was fasting as the precise estimation of the urinary N was not feasible. The amount of CH_4 produced under such conditions is negligible.

$$\text{O}_2 (4.686 + 1.23 \overline{\text{R.Q.}} - 0.707) = M \text{ kg. cal.} \quad (3)$$

where O_2 = vol. O_2 consumed in l. and

R.Q. = vol. CO_2 produced divided by vol. O_2 consumed.

Typical data and the computations of the heat production are set out in Protocol A.

PROTOCOL A

Analysis of Respiratory Exchange and the Computation of Heat Production

Ewe No. 559, Period 2A/1943. Diet 541. Daily ration 440 g.

The ration was provided at this level for 8 weeks prior to the collection period. The energy available from the ration was 1310 kg. cal./24 hr. (*vide* Table 3, per. 2A).

(1) Ventilation of respiration chamber.

Vol. passed in 24 hr.	Pre-period		Post-period	
l. dry N.T.P.	1st day	2nd day	1st day	2nd day
Metered (corr.)	34,908	34,477	34,118	32,701
Tonometer	50	50	66	66
Total	34,958	34,527	34,184	32,767

(2) Volumetric analyses of 24-hr. integral samples of air leaving chamber.

	Pre-period		Post-period	
	1st day	2nd day	1st day	2nd day
CO_2 per cent.	0.730	0.715	0.730	0.760
O_2 per cent.	20.250	20.270	20.255	20.240

Composition of ingoing air was CO_2 per cent. = 0.030
 O_2 per cent. = 20.945

(3) Respiratory exchange (from difference in composition of ingoing and outgoing air stream).

	Pre-period		Post-period	
	1st day	2nd day	1st day	2nd day
CO_2 increase per cent.	0.700	0.685	0.700	0.730
O_2 deficit per cent.	0.695	0.675	0.690	0.705

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- (4) The gravimetric estimation of CO₂ in the effluent air stream and the determination of methane by combustion of integral sample of the air leaving the chamber.

	Pre-period	Post-period
Sample vol. (48 hr.) l. dry N.T.P. (corr. <i>vide</i> text)	101.5	131.5
Carbon dioxide		
Wt. increase in absorbers g.	1.461	1.920
CO ₂ per cent.	0.731	0.743
CO ₂ increase per cent.	0.701	0.713
Methane		
Wt. increase in H ₂ O absorbers g.	0.0707	0.0811
Wt. increase in CO ₂ absorbers g.	0.0871	0.0988
CH ₄ equivalent to H ₂ O produced (corr.) in ml.	43.9	50.0
CH ₄ equivalent to CO ₂ produced (corr.) in ml.	44.2	50.2
Equivalent to CH ₄ per cent. (corr.)	0.043	0.038

- (5) Oxygen deficit, carbon dioxide and methane output in 24 hr.

	Pre-period		Post-period	
	1st day	2nd day	1st day	2nd day
O ₂ deficit, l./24 hr.	242	233	239	231
CO ₂ output, l./24 hr.	245	236	235	239
CH ₄ output, l./24 hr.	15	15	13	13

- (6) Nitrogen excretion in urine.

	Pre-period		Post-period	
	1st day	2nd day	1st day	2nd day
Mean N, g./24 hr. from 14-day collection (<i>vide</i> Table 3, per. 2A)	6.87	6.87	6.87	6.87

- (7) Heat production computed from data in (5) and (6) by means of equations (1) and (2) in text.

	Pre-period		Post-period	
	1st day	2nd day	1st day	2nd day
Non-protein respiratory quotient	0.98	0.98	0.96	1.01
	kg. cal./24 hr.			
Heat from carbohydrate and fat	1160	1115	1120	1095
Heat from protein	182	182	182	182
Combustible energy of methane evolved	142	142	123	123
Heat production	1200	1155	1180	1155

Mean heat production was taken as 1170 kg. cal./24 hr. over period of collection.

(j) *The Body Weight and Parameter for Comparison*

The assessment of the body weight of the experimental animals is, with little doubt, the most uncertain of any of the measurements involved in these experiments. In order to reduce the "inert" mass of the material in the alimentary canal to a small and reasonably constant amount, the animals were fasted and so, unless otherwise indicated in the following discussion, *W* is the sheep's body weight in kilograms, observed 96 hr. after feeding, and from which is subtracted the weight of its fleece. During the first 4 or 5 days of complete abstinence from food the sheep rarely takes a drink, and so during this time a large proportion

of the considerable volume of water in its rumen and intestine is absorbed and voided in the urine. The weight of fleece was known in these experiments—the wool grown on areas of 150 cm.² which were delineated by tattoo lines on either shoulder of each animal was collected and weighed every 14 days and the total production computed from this.

In seeking a parameter to relate the metabolic rates of experimental animals of different sizes, the surface law as expressed in Max Rubner's classic treatise "Die Gesetze des Energieverbrauchs bei der Ernährung" (1902) was set aside in favour of the exponential $W^{0.73}$. The former basis for comparison which is still in current use is seemingly a vestige of the idea that a causal relationship exists between the heat production of a homeotherm and the maintenance of its body temperature. This apparent connection between surface area (which varies exponentially with the body weight) and the respiratory exchange was appreciated and loosely defined as early as 1839 (Robiquet and Thillaye 1839) to explain the lack of any direct dependance of oxygen consumption on the body weight. There seems, however, no good reason to believe that the metabolic rate of a fasting animal in an environmental temperature within the zone of its thermic neutrality bears a closer relationship to the exponential derived from its surface area than to some other exponential of its body weight. The best observed estimations, as shown by Kleiber (1932, 1933) and by Brody and his colleagues (Brody and Procter 1932; Brody, Procter, and Ashworth 1934), indicate that both the intraspecific and interspecific relationships between body size and metabolic rate follow the same logarithmic law and that in animals ranging from the mouse to the elephant the heat productions during fasting are more uniformly proportional to the 0.73 power of their body weights than to their surface areas. The function $W^{0.73}$ *, where W is the body weight as defined above, has been employed in the following discussion as a parameter to relate the heat productions of sheep of different body weights and as a basis for comparing their behaviour with that of cattle.

III. THE RESPIRATORY EXCHANGE AND HEAT PRODUCTION OF THE SHEEP

(a) *Previous Observations*

The respiratory exchange of the sheep received attention first in Paris when Lassaigue (1846*a*, 1846*b*, 1849*a*, 1849*b*, 1849*c*, 1849*d*), who was at that time interested in ventilation, attempted to estimate the CO₂ output of a young ram confined in a hermetically sealed stall. Later Reiset (1863) conducted some convincing metabolism experiments with both rams and ewes by means of the closed-circuit Regnault-Reiset apparatus.

In Germany, Henneberg and his colleagues at Gottingen (Henneberg, Fleischer, and Muller 1873-4; Henneberg *et al.* 1869, 1870; cf. Henneberg 1870) employed the Pettenkofer apparatus, assembled at the Weende Landwirtschaftlichen Versuchsstation for the study of the metabolism of cattle, to investigate the CO₂ output of sheep. The large chamber was unsuited for the latter purpose, and in an attempt to overcome this disability two sheep at a time

*The National Research Council (U.S.A.) Conference on Energy Metabolism (1935) endorsed $W^{0.73}$ as the most suitable unit for relating body size with energy metabolism.

were observed. The CO₂ production of sheep that had fed and of two sheep that had fasted (17 hr.) were reported. Fleischer (1874) used this chamber to observe the CO₂ output of two sheep, and later Henneberg and Pfeiffer (1890) reported respiration experiments with two mature wethers to show the effect of high protein fodders on the energy expenditure. Pfeiffer (1891), after Henneberg's death, again used this apparatus in an attempt to investigate the effect of shearing on the respiratory metabolism of two young wethers. At Bonn, Hagemann (1899) healed a cannula into the trachea of a 2-year old wether and used the Zuntz-Geppert apparatus to study its metabolism. He reported the O₂ consumption and CO₂ production during six short (30 min.) interval observations of its "fasting" metabolism which were made 8½ hr. after feeding, and during similar periods observed 4 hr. after feeding. Later he rejected the cannula (Hagemann and Karpow 1906) and employed with little success a mask in conjunction with the Zuntz-Geppert apparatus to study the respiratory exchange of another sheep. Ustjanzew (1911) also depended on tracheal cannulae to study the metabolism of two wethers. Some observations (12-14 min.) were made on these animals before and after feeding on different fodders in order to investigate the *Verdauungsarbeit*;^{*} the conclusions were, however, unconvincing.

Few, if any, of the older experiments are now of more than historical interest. At the stages in the development of animal calorimetry at which they were conducted the influence of the level of feeding on the respiratory metabolism was only vaguely understood, and, as the prerequisites of a successful energy metabolism experiment were not realized, the observations rarely provided the information that was sought. In the latter part of the 19th century the more critical experiments with ruminants were conducted almost exclusively with cattle, mainly by Kellner and his students at the Mockern Landwirtschaftlichen Versuchsstation, near Leipzig, and by Armsby and his colleagues at the Institute of Animal Nutrition in Pennsylvania.

At the Tierphysiologischen Institut in Berlin, Klein and Steuber (1923*a*, 1923*b*) determined the O₂ consumption of a ram lamb during the period when it subsisted entirely on the milk of its dam and during the transition period when it was being weaned, and Klein (1923*a*, 1923*b*) in another study, observed the respiratory exchange of two older lambs.

In Russia, at the Experimental Station of the Moscow Zootechnical Institute, Schaternikoff, Moltschanowa, and Tomme (1927, 1928), while investigating the respiratory metabolism of lipoidal tissue, observed the respiratory exchange of a very fat, fat-tailed ram confined in Schaternikoff's calorimeter (1923) for two periods each of 10 hr. which began after the animal had fasted for 24 hr., and repeated the observations after surgical removal of the mass of fat on the rump. Later, Tomme (1928) published data of the respiratory exchange of a ram which had been fasted 4 days.

^{*}The mechanical work of digestion.

(b) Heat Production of the Sheep during Fasting

The heat produced by fasting sheep has been investigated in considerable detail as an integral part of Francis Benedict's comprehensive study of the relationships between the energy metabolism of different species of animals. The investigations were conducted from the Nutrition Laboratory of the Carnegie Institution of Washington, and the observations on the sheep were made at the New Hampshire Agricultural Experiment Station (Ritzman and Benedict 1930; Benedict and Ritzman 1931). A similar study of the fasting metabolism of the sheep was carried out in this laboratory (Lines and Peirce 1931; Peirce 1934). The aim of both of these investigations was to establish the "basal metabolism" of the sheep, and it was considered that this end had been achieved, but it clearly had not. Most of Benedict and Ritzman's estimations of the "standard metabolism" of sheep are derived from the respiratory exchange of groups (from 5 to 14 individuals) of animals confined in the chamber of a large open-circuit respiration calorimeter designed for cattle. The observations were made "... during three or four 30-min. periods beginning 24 hr. after the last food intake." The conditions under which the experiments were carried out were not conducive to the state of repose which is a prerequisite for critical energy metabolism experiments. The authors were aware that their "standard metabolism" figures were not those of the basal metabolism, but it was claimed that these would provide "... a measure of heat production in which the stimulatory effect which ingestion of food excites on energy production would be largely eliminated and from which the basal metabolism could be satisfactorily approximated." In summing up their findings Ritzman and Benedict stated that: "Determination of the probable basal metabolism thus involves two corrections to our standard heat production; namely, 2 per cent. for the stimulus due to food which still prevails at 24 hours after food ingestion and 15 per cent. for the effort of standing," and concluded that: "The evidence thus indicates that the standard metabolism values which are obtained here represent a uniform (*sic*) heat output which closely approximates 17 per cent. above the basal requirements in all animals except lambs which are allowed to suckle before the experiment."

The heat production of the ruminant is very materially affected by the fodder ingested 24 hr. previously, and in this respect it is distinct from that of the carnivorous animals and mixed feeders in which digestion, absorption, and the processes of intermediary metabolism responsible for the heat increment, are usually complete within 12-18 hr. after feeding. The mass of material in the paunch is not cleared each day, but, according to the nature and quantity of fodder consumed, is subject to diurnal gains and losses that determine its composition. During a period of fasting this mass continues to ferment, and for several days appreciable quantities of fatty acids arising from this activity are absorbed by the animal. The rate at which fermentation subsides to a level where the absorption of these products is so small as to no longer significantly influence the basal heat production of the animal is by no means constant: it is influenced by the previous level of feeding and by the nature of the fodder. In

consequence, the "post-absorptive state," which is an essential prerequisite in the determination of the basal metabolic rate, cannot be assumed to have been reached at any fixed interval after the ruminant has fed.

Lines and Peirce (1931), who were not convinced that the 24-hr. period of fast adopted by Benedict and Ritzman (1931) was sufficient to establish a post-absorptive state in the sheep, made their observations on "standard metabolism" after the animals had fasted for 48 hr. Their findings of the heat production of Merino sheep under these "standard conditions" were thus lower than the heat production of Durham* sheep observed by Ritzman and Benedict (1930) under the "standard conditions" of a 24-hr. fast. On these grounds, however, there is certainly no reason to assume "a distinct racial difference in that the Australian race is on the whole 13 per cent. lower than the Durham race" (Benedict 1938, p. 86). Conclusions based on differences in the heat production of sheep after 24-hr. fasting (Benedict and Ritzman 1931) are unacceptable, and those based on the "standard conditions" of Lines and Peirce (1931) are by no means secure. The "standard metabolism" of a ruminant, if expressed as the heat production of the animal at rest in an environment within the zone of its thermal neutrality, observed at any defined interval after withdrawal of food, is physiologically meaningless in the absence of a complete definition of the previous level of feeding and of the nature of the fodder. This is an obvious conclusion from the observations discussed below.

In these experiments there was a full knowledge of the prefeeding regime. The subjects were trained animals thoroughly accustomed to the environment of the calorimeters, and the heat production was observed precisely and under critical conditions over the whole 24 hr. of the fourth day of fast.

The choice of this interval of fasting was not arbitrary. Several preliminary experiments, in which the heat output of sheep was estimated at consecutive 24-hr. intervals† over periods which extended up to 12-14 days of fasting, revealed that at 72 hr. after feeding the R.Q. was usually between 0.71 and 0.70 and that the heat production had by this time reached an approximate asymptote, indicative of basal conditions, from which it receded slowly with the gradual onset of inanition. The animals employed for these preliminary experiments had previously been fed on a mixture of cereal and lucerne hay at a maintenance level.

The data relevant to the observations of the heat production during the 24-hr. period of the fourth day of fast (72-96 hr. after previous feed) are set out in Table 1.

When the intake of this particular diet (541) had been between the approximate limits of maintenance and $1\frac{1}{2}$ maintenance (periods 2, 2A, and 3), basal conditions were reached in this interval of fasting. The R.Q.'s were all close to 0.70, and the observed heat productions, when reduced to a common basis by

*The Durham strain of sheep was derived from Southdown x Rambouillet.

†Actually the 24-hr. intervals were computed from the respiratory exchange observed over $23\frac{1}{2}$ hr. of each 24 hr. The chamber was opened for $\frac{1}{2}$ hr. each day to examine the animal and to remove the excreta.

TABLE 1

THE INFLUENCE OF PREVIOUS LEVEL OF FEEDING ON THE RESPIRATORY EXCHANGE AND THE HEAT PRODUCTION DURING THE PERIOD 72-96 HR. SUBSEQUENT TO FEEDING

Period	Animal No.	W Body Wt. (kg.)	CO ₂ Increase (l./24 hr.)	O ₂ Deficit (l./24 hr.)	R.Q. (1)	S Heat Production (kg. cal./24 hr.)	$\frac{S}{W^{0.73}}$	State of Energy Balance Prior to Fast (kg. cal./24 hr.)
						(2)		(3)
1	522	31.5	112	162	0.69	750	60.5	- 405
	547	28.5	105	150	0.70	705	61.0	- 430
	558	26.0	96	138	0.70	645	60.0	- 395
	550	37.0	121	178	0.68	830	59.0	- 480
	559	36.0	122	174	0.70	810	59.0	- 500
	560	36.5	114	168	0.68	785	57.0	- 475
	572	36.0	120	171	0.70	795	58.0	- 430
2	522	30.0	126	181	0.70	845	70.5	- 20
	547	29.0	121	171	0.71	805	69.0	- 35
	558	28.0	112	165	0.68	770	67.5	- 70
	550	42.0	162	228	0.71	1060	69.0	- 30
	559	42.0	156	223	0.70	1040	68.0	- 100
	560	40.0	144	208	0.69	970	65.5	0
	572	42.0	151	216	0.70	1010	66.0	- 30
2A	522	40.0	145	208	0.70	970	65.5	+ 255
	547	40.0	152	211	0.71	990	67.0	+ 300
	550	33.5	131	187	0.70	870	67.0	+ 100
	559	31.5	133	188	0.71	880	71.0	+ 140
	560	32.5	123	176	0.70	820	66.0	+ 105
	572	30.0	128	180	0.71	835	69.5	+ 80
3	522	36.5	144	205	0.70	955	69.5	+ 250
	547	34.0	128	183	0.71	850	65.0	+ 380
	558	32.0	134	189	0.71	880	70.0	+ 230
	550	44.0	162	232	0.70	1080	68.5	+ 280
	559	46.0	169	242	0.70	1130	69.0	+ 230
	560	43.0	161	227	0.71	1060	68.0	+ 220
	572	47.0	178	250	0.71	1160	70.0	+ 270
4	522	43.0	181	244	0.74	1150	73.5	+ 780
	547	41.5	181	242	0.75	1140	75.0	+ 715
	558	39.0	172	232	0.74	1080	74.5	+ 715
	550	43.0	176	242	0.73	1145	73.0	+ 640
	559	46.5	199	265	0.75	1250	76.0	+ 730
	572	46.5	200	269	0.74	1260	76.5	+ 560

- (1) The R.Q. on fasting from low intake levels frequently falls below 0.707, due to the elimination of carbonates in the urine. Where this has occurred the R.Q. is indicated in italics.
- (2) As urinary nitrogen was not determined, the heat production was estimated directly from the observed R.Q. and the O₂ consumption according to the relationship,
 $1 \text{ litre O}_2 = 4.686 + 1.23 (\text{R.Q.} - 0.707) \text{ kg. cal.}$
- (3) These figures are $I - M$, where I = observed intake of available calories in kg. cal./24 hr. and M = the observed heat production in kg. cal./24 hr. The data from which they are drawn are set out in Tables 3 and 4.

the parameter $W^{0.73}$ varied but little about a mean of 68 kg. cal./ $W^{0.73}/24$ hr. This figure may be accepted with some confidence as a close estimate of the basal metabolism of the Merino sheep.

As the major part of the carbohydrates in diet 541 consisted of starch and sugar, fermentation in the rumen might be expected *a priori* to subside more rapidly than if the fodder had consisted essentially of more refractory fibrous materials, and so it is probable that "basal" conditions were reached in a shorter interval than they would have, had the sheep been taken from natural grazing and fasted for a similar period. But notwithstanding the relative ease with which this diet might be fermented, observed heat productions in period 4 indicate that basal conditions had not been reached after a 72-hr. period of fast from this high level of feeding. The R.Q.'s ranged about 0.74 and the mean heat production under these circumstances was 74.5 kg. cal./ $W^{0.73}/24$ hr., a figure approximately 10 per cent. above the "basal" mean. This increase in heat production is, from analysis of variance, undoubtedly real. At least one reason for the increase is indicated by the raised R.Q.'s which were above those required for a critical estimate of the basal metabolism. These prove that fermentation in the rumen was still proceeding and that the products were being absorbed at a rate which might significantly influence the heat production of the animals; but it is not clear from these figures whether this factor was the whole cause of the raised metabolism. Further investigation of this point, however, indicated (*vide infra*) that it is probable that residual fermentation in the rumen under these conditions was the sole cause of the increased heat production.

The heat productions of the individuals when they had fasted after the 10 weeks of partial starvation imposed in period 1 were in every case decidedly lower than the "basal" figures predicted from the data obtained with the same animals after the feeding conditions of periods 2, 2A, and 3. Under these circumstances the observed heat productions varied about a mean of 59 kg. cal./ $W^{0.73}/24$ hr. This lowered metabolic rate was with little doubt the effect of inanition. For 10 weeks previous to this fast the animals had received rations sufficient to provide only approximately a half of the available energy necessary to maintain them. During this period they derived and expended between 400 and 500 kg. cal. a day from their own tissues, and in consequence they had lost on an average over 2 kg. of fat and approximately 1 kg. of protein. At the time of the observations the tonus of their musculature was obviously reduced; they were weak and incapable of sustained effort.

When the experimental observations which constitute the main body of this paper were completed, five of the animals were employed in a study of the rate of change of heat production which supervenes on fasting from different levels of food intake. Three were provided with a ration of 541 sufficient to ensure an intake of available energy slightly in excess of 2 maintenance; the intakes of the others were restricted to about $\frac{1}{2}$ maintenance. All were fed at these levels for 8 weeks, after which the heat production of each was observed over successive intervals of 24 hr. which for the well-fed animals extended over

periods between 5-7 days and for the semi-starved animals from 4-6 days after feeding. The findings are set out in Table 2, and the cogent information is

TABLE 2

RESPIRATORY EXCHANGE AND METABOLIC RATE ON FASTING AFTER HIGH AND LOW LEVELS OF NUTRITION

Animal No.	Day of Fast	W Body Wt. (kg.)	CO ₂ Increase (l./24 hr.)	O ₂ Deficit (l./24 hr.)	CH ₄ (l./24 hr.)	R.Q.	M Metabolism (kg. cal./24 hr.)	M W ^{0.73} (kg. cal./24 hr.)
							(1)	(2)
522 Per. A	Fed	43.0	374	361	10.1	1.04	1840	117.0
	1	42.0	270	332	8.2	0.81	1600	103.0
	2	41.5	201	264	6.4	0.76	1240	82.0
	3	40.0	194	262	1.3	0.74	1240	82.5
	4	40.0	173	239	—	0.72	1110	75.5
	5	39.0	167	237	—	0.71	1110	76.5
522 Per. B	Fed	51.0	431	410	15.4	1.05	2100	119.0
	1	49.0	273	326	6.3	0.84	1565	92.0
	2	47.0	219	277	3.3	0.79	1320	79.5
	3	45.5	203	264	2.6	0.77	1250	77.5
	4	45.0	184	245	1.9	0.75	1160	72.5
	5	45.0	163	232	—	0.70	1080	67.5
	6	44.0	167	233	—	0.71	1090	69.0
	7	43.0	156	223	—	0.70	1040	66.5
558	Fed	41.0	340	325	10.5	1.05	1650	110.0
	1	40.0	232	264	5.8	0.88	1280	86.0
	2	39.5	217	256	4.7	0.85	1230	85.0
	3	39.0	178	232	2.3	0.77	1090	75.5
	4	39.0	167	233	2.0	0.72	1085	75.0
	5	38.0	158	221	—	0.71	1030	72.5
	6	38.5	154	217	—	0.71	1010	70.0
	7	38.0	148	212	—	0.70	990	69.5
547	Fed	44.0	364	351	8.7	1.04	1780	112.0
	1	43.5	246	280	6.0	0.88	1360	87.0
	2	43.0	214	267	5.4	0.80	1270	80.5
	3	42.5	184	243	2.8	0.76	1150	74.0
	4	42.0	177	234	2.0	0.76	1100	72.0
	5	41.0	162	228	1.3	0.71	1060	71.0
	6	41.5	154	220	1.0	0.70	1030	68.5
572	Fed	33.5	162	198	8.0	0.82	965	74.0
	1	33.0	126	161	3.1	0.78	765	59.5
	2	32.5	113	159	1.6	0.71	740	58.5
	3	31.5	106	157	0.2	0.68	730	59.0
	4	31.0	105	160	—	0.66	745	60.5
560	Fed	34.0	168	202	4.8	0.83	985	75.0
	1	33.0	120	160	2.0	0.75	755	59.0
	2	32.0	110	153	0.3	0.72	715	57.0
	3	31.5	108	155	—	0.70	725	58.5
	4	31.0	97	144	—	0.67	670	54.5
	5	31.0	107	153	—	0.70	715	58.0
	6	30.5	104	149	—	0.70	695	57.5

(1) As the urinary nitrogen was not determined, heat production was estimated directly from the observed R.Q. and the O₂ consumption according to the relationship,
1 litre O₂ = 4.686 + 1.23 (R.Q. - 0.707) kg. cal.

(2) W in this series is the observed body wt. at end of each period.

graphed in Figure 2. From these it is clear that the animals which had previously been fed at high levels had not reached basal conditions until after at least 6 days fasting; the heat production then approached the value 68 kg. cal./ $W^{0.73}/24$ hr. Thus it seems probable that the increase in the 72-96-hr. fasting metabolism observed in period 4 was due to the absorption of products arising from fermentation of residua in the rumen.

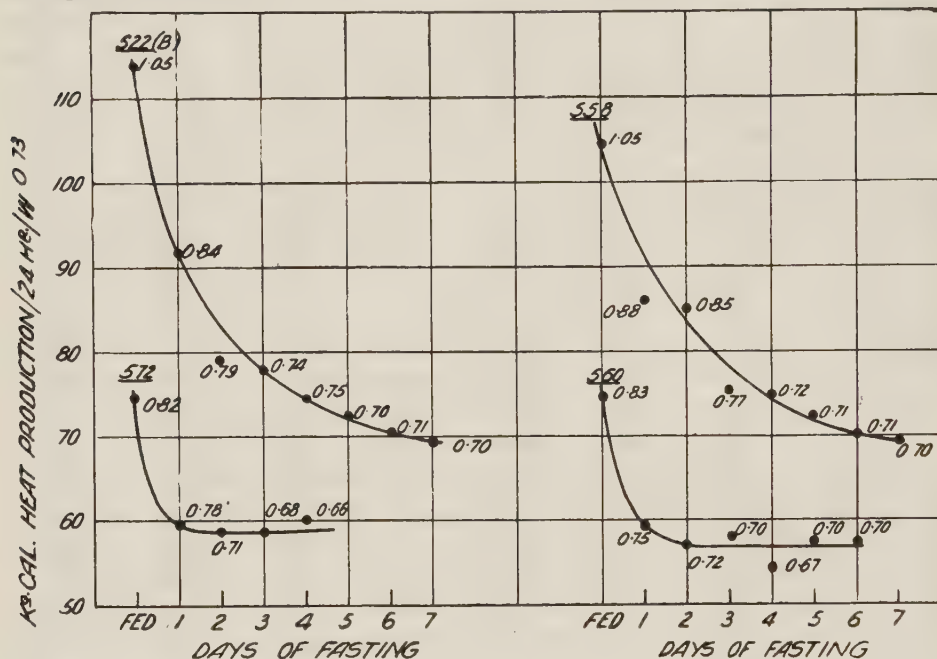


Fig. 2.—Cogent data from Table 2 are plotted to illustrate the effect of the previous nutritional level on the fasting metabolism of the sheep. It is obvious in the case of 522 (B) and of 588 that the "post-absorptive condition" which is an essential prerequisite for a critical basal metabolism determination has not been reached in the period 72-96 hr. subsequent to feeding at the high level (period 4, Tables 3 and 4) of intake of diet 541. The R.Q.'s which are shown indicate that fermentation in the rumen had not subsided in 72 hr. to a level at which the products no longer significantly influenced the basal metabolism. This explains the high heat production figures observed in period 4 (Table 1) and plotted as B" in Figure 3.

The inanition effect is obvious in the case of 572 and of 560, which had been fed at $\frac{1}{2}$ maintenance level for 8 weeks previous to the estimation. The heat production on fasting under basal conditions fell precipitately to a level considerably below 68 kg. cal./ $W^{0.73}/24$ hr., which is a critical estimate (*vide text*) of B, the basal (fasting) heat production of the sheep.

The heat production of the animals that had been fed for the previous term at the low level very rapidly fell to an asymptote markedly below the "basal" level. This inanition effect will be referred to later when the heat production of animals which had subsisted for 10 weeks at the $\frac{1}{2}$ maintenance level is discussed.

The fact that the previous level of feeding influences materially the 24-hr. and 48-hr. fasting metabolism is obvious from these experiments and calls for no further comment.

(c) Energy Transactions in the Fed Sheep

(i) *The Energy Available from Fodder 541.*—The figures relevant to the digestibility of this fodder and to the energy lost in the faeces, urine, and methane, are set out in Table 3.

From these it is clear that when fed at the submaintenance level of period 1 the proportion of the energy available from this diet was apparently reduced below the mean observed at higher levels of feeding. If the figures for period 1 are omitted the mean available energy derived from diet 541 was 70 per cent. of its gross combustible energy.

The proportion of the energy and nitrogen intake that was lost in the urine varied according to the amount of nitrogenous tissue being laid down by the animal during the period over which the estimation was made. This was influenced to some extent by the previous nutritional history of the individual. It may be recalled that some of the experimental animals were fed at increasing levels and some at decreasing levels of intake (*vide* section on experimental regime).

(ii) *Methane Production and Rumen Fermentation.*—The amount of methane evolved by the experimental animals on this diet fluctuated more erratically than is normally the case when sheep are fed on more natural diets in which the greater part of the available energy is derived from cellulose. This behaviour suggests an unstable *milieu* within the rumen. Over 50 per cent. of diet 541 consisted of the readily fermentable carbohydrates, starch, and sugars. The population of microorganisms that becomes established in the rumen when diets of this sort are fed, changes perceptibly from that which prevails when more refractory carbohydrates constitute the main substrate for fermentation. It is not improbable then that the end-products of the fermentation of starch under these conditions might differ from the dissimilation products of cellulose (Marston 1948). If this were so the heat increment quota of the available energy derived from these sources might be expected to alter according to the nature of the products. In the case of the sheep, the heat increment of the available energy from diets in which starch (grain) predominates is very definitely lower than the heat increment of the available energy from diets which consist entirely of hay from which the main ultimate sources of energy are cellulose, cellulosans, polyuronides, etc. As there is no doubt that in both cases the products of rumen fermentation are simple fatty acids, this difference in heat production presumably originates from an alteration of the ratio of these fatty acids. During the mechanical process of rumination some starch passes to the abomasum and so escapes fermentation.* From here it passes to lower levels of the intestinal

*This fact has recently been demonstrated by my colleagues, Messrs. F. V. Gray and I. G. Jarrett, who, in the course of investigating the composition of materials leaving the rumen, have observed easily detectable quantities of starch in the abomasal contents when the sheep has fed on rations containing large amounts of grain. The contents of the abomasum were collected via permanent fistulae established approximately 8 months previously.

TABLE 3

THE AVAILABLE ENERGY AND ITS DERIVATION AT DIFFERENT INTAKE LEVELS OF DIET 541

Period	Animal No.	Intake (kg. cal./24 hr.) (1)	Fæces (kg. cal./24 hr.) (2)	Urine (kg. cal./24 hr.) (3)	Methane (kg. cal./24 hr.) (4)	Available Energy (kg. cal./24 hr.) (5)	Per Cent. Available (6)	Nitrogen In Urine (g./24 hr.) (7)
1	522	729	118	49	68	495	68	5.22
	547	689	129	56	73	430	62	5.45
	558	669	120	44	60	445	66	5.00
	550	830	124	61	86	560	67	5.19
	559	794	125	71	75	525	66	4.79
	560	814	127	69	67	550	67	5.95
	572	814	129	48	90	550	67	6.10
2	522	1435	247	79	72	1035	72	5.88
	547	1395	272	70	87	965	69	5.71
	558	1414	314	60	90	950	67	5.80
	550	1778	321	94	123	1240	70	7.50
	559	1740	308	85	120	1230	71	7.80
	560	1698	257	68	114	1260	74	7.30
	572	1738	318	70	135	1220	70	7.15
2A	522	2310	475	91	120	1630	70	7.23
	547	2380	494	107	90	1690	71	7.52
	558	2200	451	63	86	1600	73	6.94
	550	1846	294	115	132	1310	71	7.46
	559	1830	306	78	133	1310	72	6.87
	560	1816	300	100	127	1290	71	7.10
	572	1714	332	81	134	1170	68	7.21
3	522	2440	476	118	146	1700	70	7.32
	547	2274	427	104	96	1650	73	7.44
	558	2274	459	96	166	1550	68	7.22
	550	2668	456	139	243	1830	69	10.45
	559	2767	539	140	156	1930	70	10.35
	560	2509	492	72	239	1710	68	9.45
	572	2710	520	118	126	1950	72	10.25
4	522	3490	679	122	94	2600	74	10.00
	547	3400	676	129	94	2500	74	9.77
	558	3235	646	110	122	2360	73	9.38
	550	3405	616	166	323	2300	68	12.10
	559	3730	774	164	170	2620	70	11.45
	572	3610	832	140	166	2470	69	11.30

(1) = the combustible energy of the fodder.

(2) = the 24-hr. mean combustible energy of the fæces passed during the 14-day collection period.

(3) = the 24-hr. mean combustible energy of the urine voided during the 14-day collection period.

(4) = the 24-hr. mean of the CH₄ produced during the 48-hr. periods before and after the 14-day collection period.

(5) = the combustible energy of the fodder minus the sum of the combustible energy of fæces, urine, and methane.

(6) = $\frac{\text{available energy} \times 100}{\text{combustible energy of fodder}}$.

(7) = the 24-hr. mean nitrogen content of the urine passed during the 14-day collection period.

TABLE 4

THE RELATIONSHIP BETWEEN HEAT PRODUCTION AND THE INTAKE LEVEL OF AVAILABLE ENERGY
FROM DIET 541

Period	Animal No.	Weight W (kg.)	Intake I (available) (kg. cal./24 hr.)	Non-protein Respiratory Quotient R	Heat Production M (kg. cal./24 hr.)	Energy Balance (kg. cal./24 hr.)	I $W^{0.73}$ (kg. cal./24 hr.)	M $W^{0.73}$ (kg. cal./24 hr.)	Energy Balance per $W^{0.73}$ (kg. cal./24 hr.)
		(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	522	31.5	495	0.80	900	- 405	40.0	72.5	- 32.5
	547	28.5	430	0.82	860	- 430	37.5	74.5	- 37.0
	558	26.0	445	0.79	840	- 395	41.0	78.0	- 37.0
	550	37.0	560	0.78	1040	- 480	40.0	74.5	- 34.5
	559	36.0	525	0.82	1025	- 500	38.5	75.0	- 36.5
	560	36.5	550	0.80	1025	- 475	40.0	74.0	- 34.0
	572	36.0	550	0.84	980	- 430	40.0	71.5	- 31.5
2	522	30.0	1035	0.90	1055	- 20	86.5	88.0	- 2.5
	547	29.0	965	0.90	1000	- 35	82.5	85.5	- 3.0
	558	28.0	950	0.89	1020	- 70	83.5	89.5	- 6.0
	550	42.0	1240	0.90	1270	- 30	81.0	83.0	- 2.0
	559	42.0	1230	0.89	1330	- 100	80.5	87.0	- 6.5
	560	40.0	1260	0.90	1260	0	85.5	85.5	0
	572	42.0	1220	0.90	1250	- 30	79.5	81.5	- 2.0
2A	522	40.0	1630	0.99	1370	+ 255	110.0	93.0	+ 17.0
	547	40.0	1690	1.00	1390	+ 300	114.0	94.0	+ 20.0
	558	36.0	1600	0.98	1355	+ 245	117.0	99.0	+ 18.0
	550	33.5	1310	0.99	1210	+ 100	101.0	93.0	+ 8.0
	559	31.5	1310	0.99	1170	+ 140	105.5	94.5	+ 11.0
	560	32.5	1290	0.98	1185	+ 105	101.5	93.5	+ 8.0
	572	30.0	1170	0.98	1090	+ 80	97.5	91.0	+ 6.5
3	522	36.5	1700	0.98	1450	+ 250	123.0	105.0	+ 18.0
	547	34.0	1650	1.00	1270	+ 380	126.0	97.0	+ 29.0
	558	32.0	1550	0.99	1320	+ 230	123.0	105.0	+ 18.0
	550	44.0	1830	0.98	1550	+ 280	116.0	98.0	+ 18.0
	559	46.0	1930	0.98	1700	+ 230	118.0	104.0	+ 14.0
	560	43.0	1710	0.96	1490	+ 220	110.0	95.5	+ 14.5
	572	47.0	1950	0.99	1680	+ 270	117.0	101.0	+ 16.0
4	522	43.0	2600	1.03	1820	+ 780	167.0	117.0	+ 50.0
	547	41.5	2500	1.04	1785	+ 715	165.0	118.0	+ 47.0
	558	39.0	2360	1.04	1645	+ 715	163.0	113.0	+ 50.0
	550	43.0	2300	1.03	1660	+ 640	148.0	106.0	+ 42.0
	559	46.5	2620	1.05	1890	+ 730	159.0	115.0	+ 44.0
	572	46.5	2470	1.02	1910	+ 560	150.0	116.0	+ 34.0

(1) W = body weight 96 hr. after feeding, reduced by weight of fleece.

(2) I = the available energy intake (cf. Table 3).

(3) R = the non-protein respiratory quotient derived by equation (1) on p. 104.

(4) M = the heat production computed by equation (2) on p. 105.

(5) The energy balance = (2) minus (4).

(6) and (7) are the intake of available calories I , and the observed metabolism M , reduced to a common basis by the parameter $W^{0.73}$.

(8) The energy balance per $W^{0.73}$ = (6) minus (7).

tract where it is hydrolysed and absorbed as simple sugars which have comparatively little effect on heat production. The proportion of the starch that escapes in this way the inevitable losses due to fermentation in the rumen is at present not clear. The evidence that is presented below indicates that the heat increment of diet 541 is unchanged at all of the observed levels of feeding, and so, if a considerable quantity of starch does escape fermentation in the rumen, then it constitutes an extraordinarily constant proportion of the total amount in the diet which is unlikely when the mechanical processes of rumination are considered. It would appear then that the amount of starch which escapes fermentation is of no great significance in the overall digestive processes and heat production of the ruminant.

(d) *The Heat Production at Different Levels of Feeding*

The data relevant to the heat production at different levels of feeding set out in Table 4 allow a complete energy balance sheet to be computed for each animal, and a precise assessment to be made of the "heat increment" which ensues when ration 541 is fed to the sheep.

As the estimations were made on animals of different body weights, and as the body weights of the individuals altered materially during the course of the experiment, the relationship of the heat production to the intake of available energy is more readily appreciated if both are reduced to a common basis by the parameter $W^{0.73}$. This procedure reduces the energy costs of the living processes (as manifested by the basal heat production) to a constant figure, and so facilitates the determination of the extra heat production which results from the energy transactions involved in the digestion and utilization of the fodder. The data treated in this way are plotted in Figure 3.

(i) *The Heat Increment of Energy Derived from Tissues during Fasting, and the Assessment of the True Basal Requirement of Energy.*—From Figure 3 there is little doubt that the relationship between the heat production, $\frac{M}{W^{0.73}}$, and the available energy, $\frac{I}{W^{0.73}}$, is linear (*vide infra*) when the animal is drawing the whole of its energy requirement from the fodder (i.e. when $\frac{I}{W^{0.73}} \geq \frac{M}{W^{0.73}}$). Extrapolation of this line to the $\frac{M}{W^{0.73}}$ axis, i.e. to the point where the animal's sole source of energy is its own tissues, should indicate the basal energy requirement of the animal. The estimated heat production at this point would be equivalent to the energy spent on the physiological requirements of living, uninfluenced by the heat increment of the materials drawn upon to provide this energy. Thus β , the "true basal energy" requirement expressed in terms of heat production, is less than B , the actual heat production under basal conditions, by the heat increment of the energy drawn from the body substance.

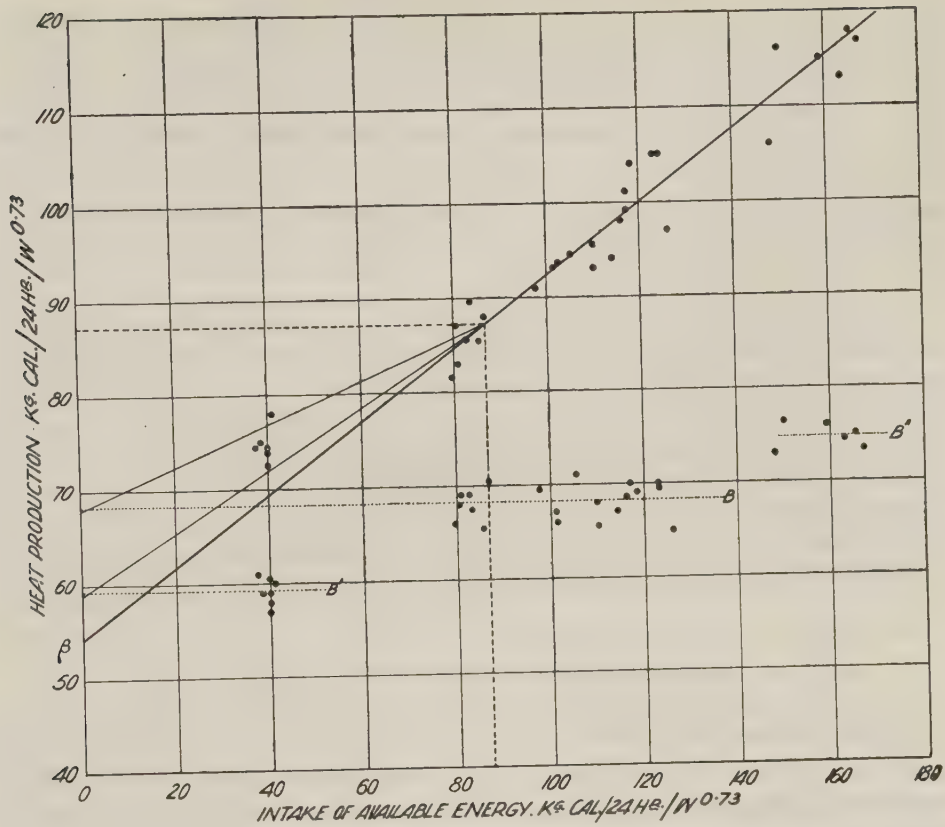


Fig. 3.—In the above figure the cogent data from Tables 1, 3, and 4 are plotted to illustrate the energy transactions of sheep fed at different levels with diet 541.

The heat production, M , and the intake of available energy, I , were reduced by the parameter $W^{0.73}$ (*vide text*) and the regression line relating them, derived by means of the equation (4) on p. 122 from the data observed when the animals had been fed at levels either close to or above their maintenance requirements. This regression has been extrapolated to the heat production axis to indicate the basal heat production, β . The fasting heat production figures (observed over the interval 72-96 hr. after feeding) are plotted at the levels of intake previous to the fast. These observations fall into three classes: B , observed in this period of fast after the feeding conditions of periods 2, 2A, and 3, may be considered a critical estimate of the "basal metabolism" of the sheep (i.e. the energy necessary to support the physiological processes plus the thermodynamic costs of deriving this energy from the body substances drawn on as an energy source during complete abstinence from food); B'' , observed on fasting after the highest level of intake, period 4, in which the post-absorptive state had not been reached after a 72-hr. fast; and B' , the fasting level observed under the conditions of partial inanition imposed by 10 weeks on the approximately half-maintenance rations of period 1. The heat production of the animal at submaintenance levels is the basal requirement of energy, β , plus the sum of the heat increment of the fodder and the heat increment of the tissue substances drawn on to make up the deficit in the basal energy requirement. This should fall between the lines joining the maintenance point on the regression with the intercept of B and B' on the heat production axis, its proximity to either of these lines being determined by the degree of inanition imposed by the previous nutritional history of the animal. The heat increment of the fodder may be determined directly from the slope of the regression.

As β estimated in this way is close to 54 kg. cal./ $W^{0.73}/24$ hr., and the observed heat production of the animal under basal conditions was close to 68 kg. cal./ $W^{0.73}/24$ hr. (*vide supra*), the heat increment is apparently close to 20 per cent. ($\frac{100(68 - 54)}{68} = 20.6$ per cent.) of the total energy drawn on and dissipated as heat during fasting. This 20 per cent. is seemingly the thermodynamic costs involved in the transfer of the materials which constitute the source of this energy into useful (productive) channels of intermediary metabolism.

(ii) *The Heat Production at Submaintenance Levels of Feeding.*—From the preceding argument it follows that the apparent (observed) heat increment of the available energy from the fodder, when the intake is insufficient to maintain energy equilibrium in the animal, is the real heat increment arising from the fodder plus the heat increment of the body substances drawn upon to make up the energy deficit. In consequence, provided the animal is not in a state of inanition, the observed heat production at submaintenance levels would be above that indicated by the regression line (Fig. 3) by a quantity of heat which should vary linearly from 14 kg. cal./ $W^{0.73}/24$ hr. (i.e. $B - \beta$) at the basal fasting level ($\frac{I}{W^{0.73}} = 0$), to nil at the maintenance level where the intake of available energy equals the energy dissipated in metabolism ($\frac{I}{W^{0.73}} = \frac{M}{W^{0.73}}$).

In the experiment under discussion the animals had been subjected for a period of 8 weeks to a level of food intake which provided less than half their maintenance requirement of energy, and so when the energy balance was determined the animals were suffering obvious effects of inanition. Their heat production on fasting was observed after 10 weeks at this level to be lowered to 59 kg. cal./ $W^{0.73}/24$ hr. The mean heat production of the fed animals at the mean intake of 39.5 kg. cal./ $W^{0.73}/24$ hr. was 74 kg. cal./ $W^{0.73}/24$ hr. Thus the observed heat production at this level of intake falls between the predicted limits of 72.5 and 76.5 kg. cal./ $W^{0.73}/24$ hr. imposed by the two lines connecting the maintenance point, $\frac{M}{W^{0.73}} = \frac{I}{W^{0.73}}$, on the regression, with the intercepts of the basal heat production level, B , and the inanition basal heat production level B' on the $\frac{M}{W^{0.73}}$ axis.

(iii) *The Heat Production Above Maintenance Levels of Feeding.*—When the intake of fodder 541 was sufficient or more than sufficient for maintenance, the heat produced by the experimental animals varied in direct proportion to the intake of available energy. Over the range studied (from close to maintenance to slightly over 2 maintenance) there was no indication that the relationship between intake of available energy and the heat output of the experimental animals varied in any regular manner from a straight line (cf. Fig. 3 where these

figures were reduced by the parameter $W^{0.73}$). The deviations of the observed heat production figures from the linear regression were normally distributed as a consequence of the experimental errors entailed in the determinations.

The obvious conclusion, then, is that *a constant proportion of the energy available from any one fodder is lost as heat increment*. Clearly an upper limit to this direct relationship between heat production and energy intake would be imposed by the maximum physiological capacity either to oxidize the fraction of the absorbed materials which gives rise to the heat increment, or to synthesize fat from the useful (productive) remainder. But it is apparent from these observations that such a limit was not even remotely approached. It is not improbable that digestion and assimilation would be severely taxed and that appetite would fail before the efficiency of the intermediary metabolic processes involved in the utilization of the absorbed materials would be impaired. When fodder 541 was offered *ad lib.*, sheep refused to consume consistently for any considerable period, more than would provide approximately two and a half times their maintenance requirements of energy, and there was nothing in their metabolic behaviour at levels closely approaching this which would suggest any falling off in the overall efficiency of utilization.

(e) *The Estimation of the Heat Increment*

In the region above maintenance, the heat increment may be computed from the heat production and available energy intake at any two points on the regression line (cf. Fig. 3), thus:

If M and M' be the heat productions at intake levels I and I' , all in kg. cal./ $W^{0.73}/24$ hr., then the heat increment of the fodder, expressed as per cent. of the available energy is $\frac{100(M - M')}{I - I'}$.

When β is known, the heat increment may be computed from any one point on the regression line above maintenance, thus:

If M kg. cal./ $W^{0.73}/24$ hr. is the heat production of the resting animal in receipt of I kg. cal./ $W^{0.73}/24$ hr. available energy, the heat increment in terms of per cent. of I is $\frac{100(M - \beta)}{I}$, or as $\beta = 54$, $\frac{100(M - 54)}{I}$.

The practical value of the latter means of estimation of the heat increment of a fodder becomes clear. If β is known precisely, only one critical determination of the heat production observed at a known level of available energy intake above maintenance is necessary for an accurate assessment of the heat increment of any foodstuff. The linear regression relating the heat production, $\frac{M}{W^{0.73}}$, and the intake of available energy, $\frac{I}{W^{0.73}}$, would invariably intercept the $\frac{M}{W^{0.73}}$ axis at the point β , and so, depending on the heat increments of the diets, these regressions would form a series of lines radiating from this point.

From the above data the heat increment of fodder 541 is 38 per cent. of the available energy. The available energy is 70 per cent. of the gross combustible energy of this fodder, and so the useful energy is 43.5 per cent. of the gross energy. As the combustible energy of the fodder is 4.50 kg. cal./g., 1 g. dry weight of the fodder would provide 1.95 kg. cal. of useful (productive) energy.

A sheep at rest in an environmental temperature within the zone of its thermal neutrality requires the useful energy equivalent of β , i.e. 54 kg. cal./ $W^{0.73}$ /24 hr. to maintain it. Any excess of useful energy fed over this quantity will be retained by the animal in the form of protein, fat, or carbohydrate, or may be used for production. If the quantity of useful energy provided by the fodder is insufficient for maintenance, the animal will lose energy and the amount of this loss will be equivalent to 125 per cent. of the useful energy deficit—the heat increment of the tissue substances drawn upon being 20 per cent. (*vide supra*).

(f) *The Significance of β , the True Basal Energy Requirement for Maintenance*

Theoretically, within the limits of applicability of the $W^{0.73}$ exponential relationship between metabolic rate and body weight, the value of β should be identical for all homeothermic animals.

There is only one set of independent observations in the scientific literature that provides suitable data for testing this hypothesis. Forbes and his colleagues (Forbes *et al.* 1928, 1930) carried out, in Pennsylvania, a fine series of critically conceived and meticulously observed determinations of the heat output of 4 steers fed on rations composed of 50 per cent. lucerne hay and 50 per cent. grain (maize), at levels which, for most of the observations, ranged between $\frac{1}{2}$ maintenance and 2 maintenance, two observations being made at $2\frac{1}{2}$ maintenance, and in one case at the very high level of 3 maintenance. The animals were fed at each level for 10 days before 18-day collections of the visible excreta were made, and the heat dissipated by the animals was determined by direct calorimetry immediately after the collection period.

The reported figures of body weight, W , the available energy intake, I , and the observed heat production, M , of these 4 steers at the levels above maintenance have been computed to the base $W^{0.73}$ and plotted in Figure 4, alongside the data from the sheep. The regression lines in both cases were determined as follows:

Let $\frac{M}{W^{0.73}} = y$, the dependent variable, and $\frac{I}{W^{0.73}} = x$, the independent variable, and Y_i be the predicted value of y when $x = x_i$. The coefficients in the regression equation

$$Y_i = \bar{y} + b(x_i - \bar{x}) \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

were determined by the method of least squares and are given by the arithmetic mean, \bar{y} , and the regression coefficient,

$$b = \frac{\sum (x - \bar{x}) (y - \bar{y})}{\sum (x - \bar{x})^2}$$

On either side of the plotted regressions in Figure 4 the confidence limits at a probability $P = 0.05$ are indicated. These were derived as follows:

The variate y is assumed to be normally distributed about Y with constant variance σ^2 . Since Y is a linear function of normally and independently distributed quantities,

$$\begin{aligned} V(Y_i) &= V(\bar{y}) + V(b) (x_i - \bar{x})^2 \\ &= \frac{\sigma^2}{n} + \frac{(x_i - \bar{x})^2 \sigma^2}{\sum (x - \bar{x})^2}, \end{aligned}$$

the symbol V designating variance.

Substituting for σ^2 , its estimate s^2 derived from $s^2 = \frac{\sum (y - Y)^2}{(n - 2)}$, the estimated variance of

$$Y_i = s^2 \left\{ \frac{1}{n} + \frac{(x_i - \bar{x})^2}{\sum (x - \bar{x})^2} \right\}$$

and the limits are given by the hyperbolae

$$Y = \bar{y} + b(x - \bar{x}) \pm ts \left\{ \frac{1}{n} + \frac{(x - \bar{x})^2}{\sum (x - \bar{x})^2} \right\}^{1/2} \quad (5)$$

where the statistic, t (Fisher 1925) has $(n - 2)$ degrees of freedom and is taken from the chosen level of probability.

The data from the experiments of Forbes and his colleagues on the steer, treated in this way, gave a value of $\beta = 51.85$ with a standard deviation estimated approximately to be 3.38; the data from the observations on the sheep discussed above gave a value of $\beta = 54.56$ with a similarly estimated standard deviation of 2.30.

From this it may be concluded that the true basal energy requirements of the steer and the sheep are related exponentially by a parameter to which $W^{0.73}$ is a close approximation, and that within the experimental error involved in its determination the value of β derived in the above manner is close to 54 kg. cal./ $W^{0.73}/24$ hr. This conclusion is quite independent of actual observations of basal (fasting) heat production.

As there is no reasonable doubt that, above maintenance, the heat production/available energy relationship is linear, the intercept, β , at which the extrapolated regression cuts the heat production axis should indicate the true basal requirement of energy, provided that the heat increment of the fodder is the same above and below the maintenance point. The slope of the regression above this point might be expected *a priori* to be increased by the thermodynamic costs involved in the conversion of carbohydrate to fat. Existing knowledge of the course that this conversion takes *in vivo* is not precise, but the estimate that the free energy of the reaction is 6.3 per cent.* of the calorific value of the glucose converted is probably a close approximation.

*This figure was computed by Bleibtreu (1901) from a hypothetical equation which assumed the maximum conversion of glucose to palmitic acid. It is employed in computing the heat production from the respiratory exchange when the R.Q. exceeds 1.00 (cf. Lusk 1915, 1928).

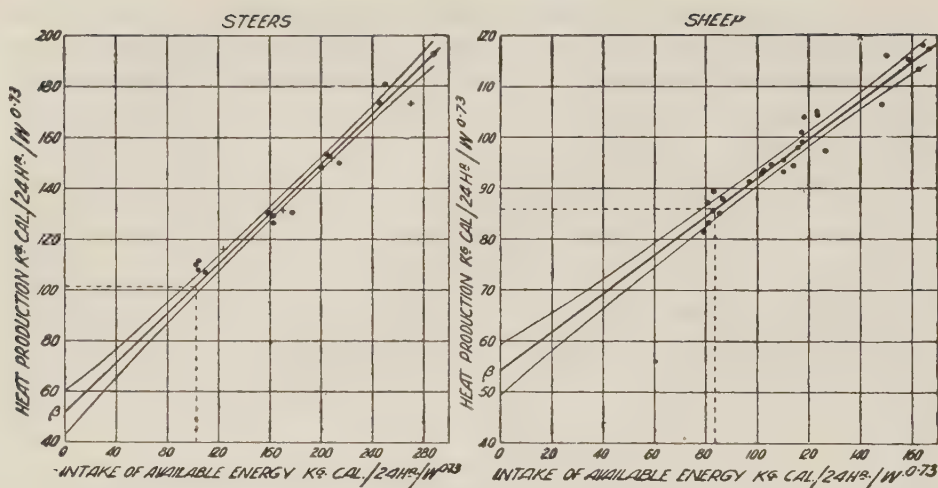


Fig. 4.—In the above figure the regressions relating the intake of available energy with the heat production were derived by the means set out on p. 122. The data which refer to steers (oxen) were computed from the observations of Forbes and his colleagues (Forbes *et al.* 1928, 1930), who measured in the direct calorimeter the heat production of 4 steers after they had fed on rations composed of 50 per cent. lucerne hay and 50 per cent. maize at levels which ranged between maintenance and the very high level of 3 maintenance. The observations that Mitchell *et al.* (1932) made on an ox are shown as crosses. These were not included in the calculations as the diet was slightly different from that employed by Forbes *et al.* The data which refer to sheep are those set out in Tables 3 and 4. The confidence limits plotted on either side of each regression indicate that the probability is $P = 0.95$ that this relationship will lie within these limits, and are given by the hyperbolae defined by expression (5) on p. 123. The data on which the calculations relating to cattle were based are the available energy, the observed (direct) heat output uncorrected for the energy expended in standing, and the observed body weight. These were reduced by $W^{0.73}$, W here being the body weight of the fed animal and not, as in the case of the sheep, the body weight of the animal after it had fasted 3 days. For the observations on the steers $n = 12$ and $t = 2.179$ and 3.055 at the respective probabilities $P = 0.05$ and 0.01 ; for the observations on the sheep $n = 25$ and $t = 2.060$ and 2.787 at the respective probabilities $P = 0.05$ and 0.01 .

Because the composition of the rations consumed by the steers was not identical with diet 541 which was fed to the sheep, the slopes of the regressions differ according to the heat increment of the fodder. The aim of the above comparison, however, is not concerned with the slopes of these regressions but with the points at which they intercept the $\frac{M}{W^{0.73}}$ axis, which indicate the heat production arising from the energy spent in the animal organism by its basic living processes. The value of this "true basal metabolism" has been designated β . From the observations presented in this paper $\beta = 54.56$ with a standard deviation estimated approximately as 2.30; from the observations of Forbes *et al.* $\beta = 51.85$ with a standard deviation of 3.38 similarly estimated. There is no significant difference between these values. A close estimate of the "true basal metabolism" is thus 54 kg. cal./ $W^{0.73}$ /24 hr. (*vide text*). This implies that the fasting metabolism of the sheep under basal conditions (68 kg. cal./ $W^{0.73}$ /24 hr.) is subject to approximately a 20 per cent. heat increment.

If this 6.3 per cent. is part of the observed heat increment above maintenance, the estimation of the true basal metabolism from β would be approximately 6 per cent. high. There is no evidence, however, that the synthesis of fat by the ruminant adds materially to the other costs of utilization of the nutrients which constitute the source of available energy. These, for most part, are simple fatty acids produced, at an expense of approximately 6 per cent. of the combustible energy of the substrate, by fermentation of carbohydrates (Marston 1948), and the energy dissipated in the process of fat production from the "useful energy" of these fermentation products is probably a very small fraction of the free energy of the conversion of carbohydrate to fat. Thus, in ruminants there is no cogent physiological or thermochemical basis which should lead to the conclusion that the slope of the regression would change at the maintenance point because of an increased heat increment due to fat formation.

The heat increment above maintenance might, however, be expected to be influenced under certain circumstances when the animal produces relatively large quantities of protein by reassembling and condensing the amino-acids arising from hydrolytic cleavage of protein in the fodder. The thermodynamic costs of these transactions are small compared with those involved in the katabolic destruction and utilization of protein as an immediate source of energy or for fat formation. The circumstances of lactation, rapid growth, or wool production, would thus tend to decrease the heat increment by a quantity equivalent to the specific dynamic effect of protein, retained as such or secreted, minus the energy costs involved in the synthesis of protein from amino-acids.

IV. THE PHYSIOLOGICAL BASIS OF CURRENT FEEDING STANDARDS

The standards of current feeding practice are based on two main systems that have been used for the evaluation of the capacity of fodders to provide energy to ruminants—the Net Energy Principle of H. P. Armsby, which is employed in the United States, and the Starch Equivalence System of Oskar Kellner, which is employed in continental Europe and in the United Kingdom.

It is not proposed here to discuss in detail the relative merits of these guides to practical husbandry, but to examine briefly, and in light of the above findings, the physiological foundations upon which they are based. Both investigators employed cattle as experimental animals; the former used a direct calorimeter and the latter an indirect (respiration) calorimeter for the determination of heat production.

Armsby estimated the value of fodders in terms of their capacity to prevent loss of body substance, and so his observations were made at submaintenance levels of feeding. Kellner evaluated fodders in terms of their capacity to provide for fat formation, and so observed the heat production of animals that had been fed at levels above maintenance. As the basic findings were not in agreement, many attempts have been made to reconcile the two sets of data. An explanation is suggested as a corollary from the observations of Forbes and his

colleagues (*loc. cit.*). Using the fasting heat production as a base, Forbes concluded that "the heat production increased slowly between fasting and maintenance and much more rapidly above maintenance," and the fact that the metabolic phenomena below the maintenance level are influenced by the heat increment of the energy source drawn from the body was recognized in the conclusion of Forbes and Kriss (1932) "that the curvature of the line representing the relation of heat production to food consumption between fasting and maintenance is determined by the excess of the waste heat of utilization of increasing food nutrients as compared with that of decreasing nutrients katabolized."

The observations of Kellner are thus more simple to interpret than those of Armsby, whose computations were complicated by the apparent change in the value of the heat increment below the maintenance point which is a consequence of the use of the fasting metabolism as a base of reference.

Wiegner and Ghoneim (1931) have attempted to interpret this phenomenon by assuming that the net energy of the fodder changes with the level of feeding, and that the magnitude of this change decreases from a maximum at zero intake to nil when the maximum efficacy (*Hochstwirkung*) is reached. Their equation of this relationship,

$$\frac{dA}{dF} = K(H - A)$$

in which A = net energy, F = the available energy, H = the maximum net energy of the fodder, and K = an efficiency constant independent of the plane of nutrition, which assumes that $\frac{dA}{dF}$ decreases continuously as the food intake increases from the fasting level, is physiologically meaningless, and the attempts, by empirical choice of constants, to fit this type of curve to the well-observed data of Forbes and his colleagues are not convincing.

From the experimental observations on the sheep discussed above there is no evidence that $\frac{dA}{dF}$ changes over the range of F from $\frac{1}{2}$ maintenance to 2 maintenance, the apparent alteration below maintenance being due to the heat increment of the body substances drawn upon to make up the energy deficit.

V. GENERAL CONCLUSIONS

The above findings are in general accord with Rubner's (1902) theory of energy metabolism which, *inter alia*, was based on the thermodynamics of intermediary metabolism. The specific dynamic effect (heat increment) of ration 541 was, within the experimental error involved in its determination, unchanged at different levels of feeding. The fact that it was a large proportion of the available energy implies strongly that the transfer of some at least of the absorbed materials, that constituted the energy source, into the cycles of intermediary metabolism involved very considerable thermodynamic costs, which overall were close to 38 per cent. of the energy available from the fodder at all levels of feeding.

The magnitude of the heat increment arising from the carbohydrate fermentation products that constitute the main energy source of the fed ruminant exceeds very materially the specific dynamic effect of simple sugars from which mixed feeders derive the greater proportion of their energy, and it is greater than the S.D. effect of protein with its contingent costs of elimination of the nitrogenous residues. The fermentation process *per se* is in part responsible for an addition to the heat increment, but it is unlikely that the free energy of the microbiological dissimilation of carbohydrate within the paunch greatly exceeds 6 per cent. of the combustible energy of the carbohydrate transformed (Marston 1948), and this would account for only about 15 per cent. of the heat increment. It would appear then that the contribution made by the energy costs of utilization of some or all of these simple fatty acids is the main factor which determines the large heat increment of the energy available to the ruminant from its fodder.

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It is also a pleasant duty for the author to acknowledge again the stimulating interest of his teacher, Sir Charles Martin.

VII. REFERENCES

- ANDERSEN, A. C. (1920).—*K. VetHojksk, Aarsskr.* 157-79.
——— (1922).—*Biochem. Z.* 130: 143-50.
ARMSBY, H. P. (1917).—"The Nutrition of Farm Animals," pp. 279-80, (Macmillan Co.: New York.)
——— and FRIES, J. A. (1903).—U.S. Dept. Agric., Bur. Anim. Ind., Bull. No. 51, 77 pp.
BAILEY, C. V. (1921).—*J. Lab. Clin. Med.* 6: 2-24.
BENEDICT, F. G. (1938).—Carnegie Inst., Wash., Publ. No. 503, p. 86.
——— and RITZMAN, E. G. (1931).—*Arch. Tierernahr. Tierz.* 5: 1-88.
BLEIBTREU, M. (1901).—*Pflug. Arch. ges. Physiol.* 85: 345-400.
BRODY, S., and PROCTER, R. C. (1932).—Univ. Missouri Agric. Exp. Sta., Res. Bull. No. 166: pp. 89-101.
———, ———, and ASHWORTH, U. S. (1934).—*Ibid.* No. 220, 40 pp.
CARPENTER, T. M., FOX, E. L., and SEREQUE, A. F. (1929).—*J. Biol. Chem.* 83: 211-30.

- FISHER, R. A. (1925).—"Statistical Methods for Research workers." (Oliver and Boyd: Edinburgh.)
- FLEISCHER, M. (1874).—*J. Landw.* 22: 265; Tagebl. d. 46 Vers. dtsch. Naturforsch. u. Ärzte in Wiesbaden 1873, 113.
- FORBES, E. B., BRAMAN, W. W., and KRISS, M., with the collaboration of JEFFRIES, C. D., SWIFT, R. W., FRENCH, R. B., MILLER, R. C., and SMYTHE, C. V. (1928).—*J. Agric. Res.* 37: 253-300.
- , ———, and KRISS, M., with the collaboration of SWIFT, R. W., FRENCH, R. B., SMYTHE, C. V., WILLIAMS, P. S., and WILLIAMS, H. H. (1930).—*Ibid.* 40: 37-78.
- and KRISS, M. (1932).—*J. Nutrit.* 5: 183-97.
- and VORIS, LE R. (1943).—*Ann. Rev. Physiol.* 5: 105-22.
- HAGEMANN, O. (1899).—*Arch. Anat., Physiol. Abt., Suppl. Bd.* pp. 111-40.
- and KARPOW, M. S. (1906).—*Landw. Jb.* 35, Erg.-Bd. 4: 371-402.
- HENNEBERG, W. (1870).—"Neue Beiträge zur Begründung einer rationellen Fütterung der Wiederkäuer" 1: 5 *et seq.* (Göttingen.)
- , FLEISCHER, M., and MÜLLER, K. (1873-4).—*Jber. AgrikChem.* 16-17 (II): 145.
- , KUHN, G., MARCKER, M., SCHULZE, E., and SCHULTZE, H. (1869).—*J. Landw.* 17: 176, 277, 409.
- , ———, MARCKER, M., SCHULZE, E., and SCHULTZE, H. (1870).—*Ibid.* 18: 40.
- and PFEIFFER, TH. (1890).—*Ibid.* 38: 215-78.
- KELLNER, O. (1905).—"Die Ernährung der landwirtschaftlichen Nutztiere." (P. Paray: Berlin.)
- and KOHLER, A. (1900).—*Landw. VersSta.* 53: 1-474.
- KLEIBER, M. (1932).—*Hilgardia* 6: 315-53.
- (1933).—*Biederm. Zbl. B.* 5: 1-12.
- KLEIN, W. (1923a).—*Berl. tierarztl. Wschr.* 39: 159-62.
- (1923b).—*Ibid.* 39: 231-4.
- and STEUBER, M. (1923a).—*Biochem. Z.* 136: 477-81.
- , ——— (1923b).—*Ibid.* 139: 66-73.
- LASSAIGNE, J. L. (1846a).—*J. Chim. Med.* 22: 477-84.
- (1846b).—*Ibid.* 22: 751-8.
- (1849a).—*Ibid.* 25: 13-14.
- (1849b).—*J. Prakt. Chem.* 46: 287-92.
- (1849c).—*J. Chim. Med.* 25: 253-7.
- (1849d).—*J. Prakt. Chem.* 47: 136-8.
- LINES, E. W. L. (1938).—*J. Agric. Sci.* 28: 663-78.
- and PEIRCE, A. W. (1931).—*Coun. Sci. Industr. Res. Aust., Bull. No. 55*, 34 pp.
- LOEWY, A. (1911).—*Der Gaswechsel der Organe und Gewebe. Oppenheimers Handbuch der Biochemie des Menschen und der Tiere. Vol. 4: pp. 1, 279.* (G. Fischer: Jena.)
- LUGG, J. W. H. (1938).—*J. Agric. Sci.* 28: 688-94.
- LUSK, G. (1915).—*J. Biol. Chem.* 20: 555-67.
- (1928).—"The Science of Nutrition." 4th Ed. (W. B. Saunders: Philadelphia.)
- MAGNUS-LEVY, A. (1907).—"The Physiology of Metabolism." Anglo-American issue under the editorship of I. W. Hall. 452 pp. (Chicago). In NOORDEN, C. VON.—"Metabolism and Practical Medicine." Vol. 1.

- MARSTON, H. R. (1935).—*J. Agric. Sci.* 25: 103-12.
——— (1948).—*Biochem. J.* (In press.)
- MERING, J. VON, and ZUNTZ, N. (1877).—*Pflug. Arch. ges. Physiol.* 15: 634-6.
- MITCHELL, H. H., and HAMILTON, T. S., with the technical assistance of MCCLURE, F. J., HAINES, W. T., BEADLES, J. R., and MORRIS, H. P. (1932).—*J. Agric. Res.* 45: 163-91.
- MOLLGAARD, H. (1929).—"Futterungslehre des Milchviehs. Die quantitative Stoffwechselformung und ihre bisherigen Resultate beim Milchvieh." (M. & H. Schaper: Hanover.)
- PEIRCE, A. W. (1934).—*Coun. Sci. Industr. Res. Aust., Bull. No. 84*, 22 pp.
- PFEIFFER, TH. (1891).—*J. Landw.* 39: 1.
- REISET, J. (1863).—*Ann. Chim. Phys.* 3 ser, 69: 129-69.
- RITZMAN, E. G., and BENEDICT, F. G. (1930).—*Univ. New Hampshire, Agric. Exp. Sta., Tech. Bull. No. 43*, 23 pp.
- ROBIQUET, I., and THILLAYE, E. (1839).—*Bull. Acad. Roy. Med., Paris* 3: 1094-100.
- RUBNER, M. (1902).—"Die Gesetze des Energieverbrauchs bei der Ernährung." (F. Deuticke: Leipzig, und Vienna.)
- SCHARLING, E. A. (1843).—*Ann. Chem. Pharm.* 45: 214-42; reprinted in detail in *Ann. Chim. Phys.* 3 ser, 8: 478.
- SCHATERNIKOFF, M. N. (1923).—*Pflug. Arch. ges. Physiol.* 201: 56-9; *J. Biol.* 1: 148 (1925).
———, MOLTSCHANOWA, O. P., and TOMME, M. TH. (1927).—*J. Biol. Med. Exp., Moscow* 7: 375-82. (In Russian.)
———, ———, ——— (1928).—*Pflug. Arch. ges. Physiol.* 218: 216-21.
- SEGUIN, A., and LAVOISIER, A. L. (1789).—*Mem. Acad. Sci., Paris*, p. 185.
- SMITH, E. (1859a).—*Philos. Trans.* 149: 681-714.
——— (1859b).—*Ibid.* 149: 715-42.
- TOMME, M. TH. (1928).—*Exp. Sta. Moscow Zootech. Inst. Bull. No. 9* (in Russian).
- USTJANZEW, W. (1911).—*Biochem. Z.* 37: 457-76.
- WIECNER, G., and GHONEIM, A. (1931).—*Biederm. Zbl.*, B 2: 193-232.
- ZUNTZ, N., and SCHUMBURG, H. (1901).—*Studien zur Physiologie des Marsches. Bibl. v. Coler* 6: 1-361. (Hirschwald: Berlin.)

THE PRODUCTION OF METHANE AND HYDROGEN BY THE SHEEP

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Summary

The rate of production of methane by sheep and the effects of fasting and re-feeding on production of methane and hydrogen were studied.

The production of methane by sheep fed at regular intervals of 24 hours reached a peak in the first four hours after feeding, and fell during the remainder of the day.

Production of methane ceased after the animal had fasted about 4 days. On resumption of feeding the formation of combustible gas is influenced by the duration of the fast. In animals fed after prolonged fasts, or after emptying the rumen, free hydrogen was observed as an initial product, being gradually replaced by methane. Inhibition of such hydrogen formation was brought about by addition of normal rumen contents from another animal.

These observations are in conformity with the hypothesis that at least two organisms are involved in the production of methane, one producing hydrogen and another catalysing the reduction of carbon dioxide to methane by the hydrogen so formed.

I. INTRODUCTION

As the energy represented by the combustible gases evolved during fermentation in the intestinal tract constitutes a considerable proportion of the energy of the digestion products, the precise estimation of methane, which is the combustible gas normally produced, is essential when considering the energy transactions of ruminants.

The literature on methane production has been reviewed in recent years by Washburn and Brody (1937) and by Cole *et al.* (1945). Small amounts of free hydrogen have been reported occasionally to be present in gas samples taken direct from the rumen by workers who have employed volumetric methods for the estimation. These analyses, however, are not completely convincing. The analytical procedure is liable to error, and from the many measurements made of the inflammable gases produced by sheep confined in the calorimeter chambers in this laboratory, it appears that methane is the only combustible gas produced under normal feeding conditions. These findings agree with those of other workers who have employed gravimetric methods for the determinations.

The initial object of the investigations reported here was to determine the diurnal variations in the rate of methane production by sheep which were fed once each day, and to determine the effects of fasting and of subsequent refeeding. In the course of the experiments a large proportion of free hydrogen was

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observed in the gases evolved when feeding was resumed after a fast of several days, and the investigations were therefore extended to examine the conditions under which hydrogen is produced.

II. EXPERIMENTAL PROCEDURE

The experiments were divided into two series according to the method of estimation of methane and hydrogen. In the first series the gaseous metabolism of the sheep under observation was measured by the open circuit method in the apparatus which has been described by Lines (1938). Methane and hydrogen were determined by the combustion of an aliquot of about 0.17 per cent. of the ventilation stream, using the method described by Lugg (1938). The sample for 24-hour estimations usually amounted to about 60 litres. When the duration of the sampling was 12 hours or less, the rate of flow through the combustion chain was doubled in order to obtain sufficient water and carbon dioxide for precise estimation. The methane concentration of the sample was computed after deducting the small blank due to the combustible gases in normal air, assumption being made that the ratio of the weights of water and carbon dioxide arising from complete combustion of methane was as 36:44. When water was produced in excess of this proportion a corresponding amount of free hydrogen was presumed to be present.

In the second series of experiments, gas samples were withdrawn directly from the rumen either through an established fistula, using a suitably adapted stopper, or through a catheter passed into the rumen by means of a trochar and cannula and left *in situ* for the duration of the experiment. Gas samples were withdrawn by a water-lubricated 50-ml. syringe, precautions being taken to prevent any leakage of air. The gas was transferred immediately to a Bailey (1921) gas bottle and subsequently analysed volumetrically in the apparatus of Shepherd (1931). In this method a H:C ratio characteristic of methane is indicated after the combustion by a correct relationship between the total contraction, the volume of carbon dioxide produced and the volume of oxygen consumed. When both methane and hydrogen were present the volume of methane was assumed to be equal to the volume of carbon dioxide produced, while the volume of hydrogen was obtained by subtracting the volume of oxygen used from the total contraction.

In both series of experiments the daily diet consisted of a mixture of 500 g. of chaffed cereal hay and 500 g. of chaffed lucerne hay. This amount was consumed completely, usually within 1-2 hours, by sheep which were fed at regular 24-hourly intervals and by those fed after short periods of fasting. However, after longer periods of fasting, the intake by individual sheep was observed to vary considerably, some eating 60 per cent. of the diet in the first 24 hours and reaching 100 per cent. intake within 3 days, while others ate as little as 10 per cent. on the first day and did not consume the whole of their ration on any day during the period of experiment, usually 5 days. Water was available *ad lib.* at all times.

III. EXPERIMENTAL RESULTS

(a) Production of Methane after Normal Feeding

The production of methane by four sheep fed as described above was measured in the respiration chamber for six successive periods of four hours each. Methane was the only combustible gas observed in these experiments. Its rate of production during the four-hour periods is shown in Table 1.

TABLE 1
PRODUCTION OF METHANE IN SIX SUCCESSIVE PERIODS OF FOUR HOURS

Hours after Feeding	Methane (Litres per Hour)				Mean Per Cent. of 24-Hour Total
	Animal P. 1	Animal P. 1	Animal P. 2	Animal P. 3	
0-4	1.07	0.67	0.44	0.42	34
4-8	0.66	0.39	0.37	0.27	22
8-12	0.35	0.17	0.36	0.18	14
12-16	0.26	0.22	0.22	0.18	11
16-20	0.21	0.21	0.19	0.14	10
20-24	0.17	0.19	0.13	0.18	9

The most rapid production in each case occurred during the first 4 hours, after which a fairly regular decrease is evident.

In three further experiments the composition of rumen gas was examined by analysing samples withdrawn from the animal at intervals. Similar results were obtained from each experiment, and the data for one of them are plotted in Figure 1. The carbon dioxide content of the gas was high in the early stages of fermentation; it decreased later in the day while the proportion of methane

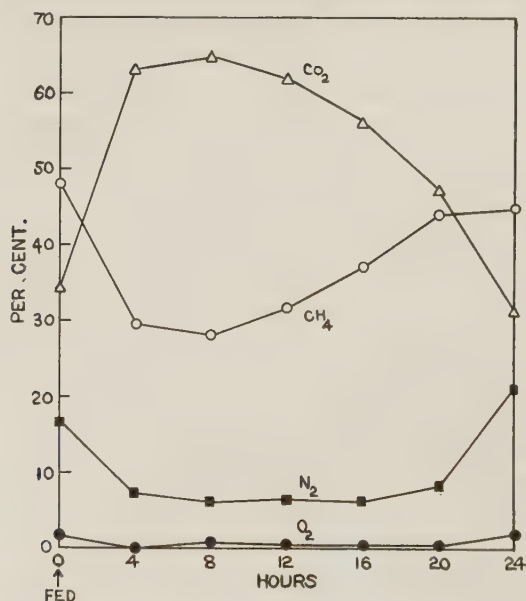


Fig. 1.—Diurnal variation in the composition of rumen gas. Samples withdrawn at 4-hourly intervals commencing just before feeding.

increased. These proportions obviously were influenced by the relative rates of carbon dioxide and methane production; they provided no indication of the absolute rate of methane production. Considerable quantities (3-30 per cent.) of nitrogen and small quantities (0-7 per cent.) of oxygen were present in the rumen gas during the diurnal cycle. At the height of fermentation, oxygen was displaced almost completely from the gases above the rumen contents.

(b) *Production of Hydrogen and Methane during and after Fasting*

Five animals were fasted for varying periods up to 15 days in the respiration chamber and the production of methane determined by the first procedure. It was found that methane production ceased within 3-5 days in all cases and that no hydrogen was present in the ventilation stream.

Three animals were fasted until methane could no longer be detected. On refeeding, recovery of methane production was complete in 3-4 days. No hydrogen appeared under these conditions.

Figure 2 illustrates both the above phases in one of these animals which was fed after a 4-day period of fasting.

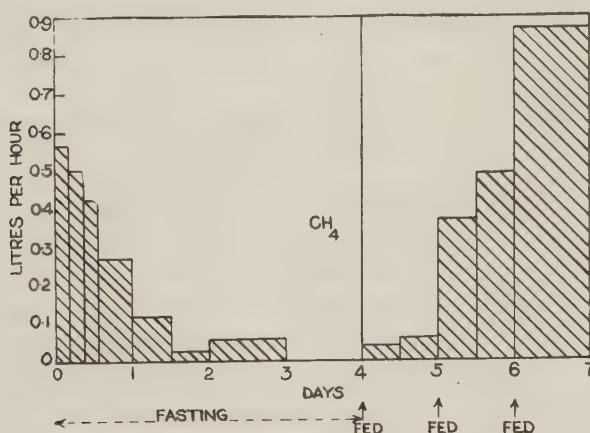
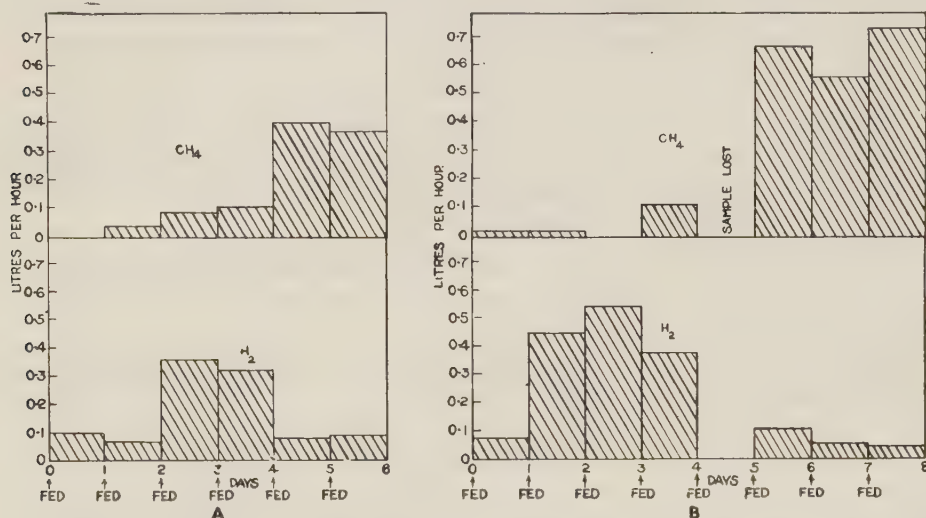


Fig. 2.—Production of methane by a sheep fasting for 4 days then fed for 3 days.

Very different results were obtained on refeeding animals after they had fasted for more prolonged periods (13-15 days). Frequently a delay of 3-4 days was observed before methane production reached even half its normal level, and *in these cases free hydrogen appeared* as an initial product of fermentation. This production of hydrogen reached a maximum usually about the third day of feeding, after which it waned and disappeared in the subsequent few days, by which time methane production had reached normal values. Sixteen experiments of this type were carried out. In five cases hydrogen reached concentrations of 0.2-0.5 litre per hour; in five others concentrations of 0.05-0.2 litre per hour were observed, while in the remaining six experiments hydrogen was detected only in small amounts (less than 0.05 litre per hour).

The experiments set out in Figures 3A and 3B, which are typical of the first group, show the methane and hydrogen production after a fast of 15 days. The data in Figure 3B are not complete owing to the accidental loss of the sample on the fifth day, but the phenomenon is strikingly evident.



Figs. 3A and 3B.—Production of methane and hydrogen by a sheep fed for 6 days (Fig. 3A) and 8 days (Fig. 3B) after 15 days fasting.

Confirmation of the production of hydrogen after long fasting periods was obtained by the use of the direct sampling method. Four experiments were carried out in which samples of rumen gas were withdrawn at intervals during a prolonged fast (7-13 days) and for several days after feeding was resumed. The data shown in Figure 4 are typical of these experiments. After the third day of fasting, the rumen gas in this animal remained very constant in composition; it

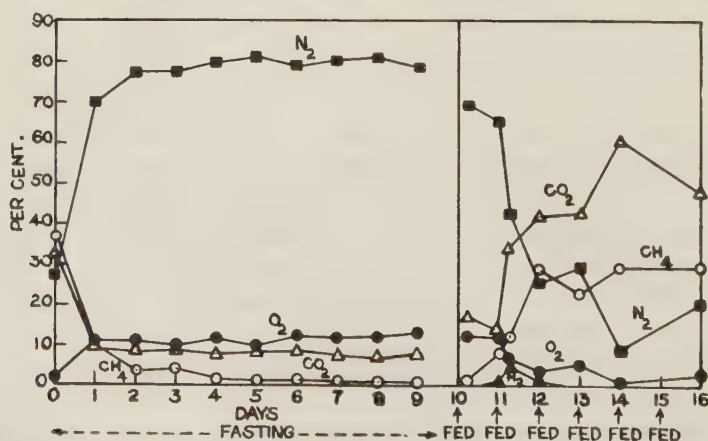


Fig. 4.—Composition of rumen gas in a sheep fasting for 10 days then fed for 6 days.

consisted largely of nitrogen. When feeding was resumed after the tenth day, a small amount of hydrogen was found to be present for the following two days, during which period the methane concentration returned to normal.

The formation of hydrogen after prolonged fasting was not as strikingly demonstrated by the direct sampling procedure as it was by estimating the total quantities evolved (cf. Figs. 3A and 3B). In the next section experiments are described in which artificial emptying of the rumen was used in place of long fasting, and in these cases both methods gave unequivocal results.

(c) *Production of Hydrogen and Methane after emptying the Rumen*

It was thought that the effect of a long fasting period could be simulated by emptying the rumen. Animals with rumen fistulae were fasted for 3 days and at the end of each of the second and third days the rumen was washed out with warm water and the contents replaced by 2-3 litres of N/5 sodium bicarbonate.

Three animals which were treated in this way were placed in the respiration chamber, fed, and the hydrogen and methane production measured. Data from one of these experiments shown in Figure 5 indicate the production of con-

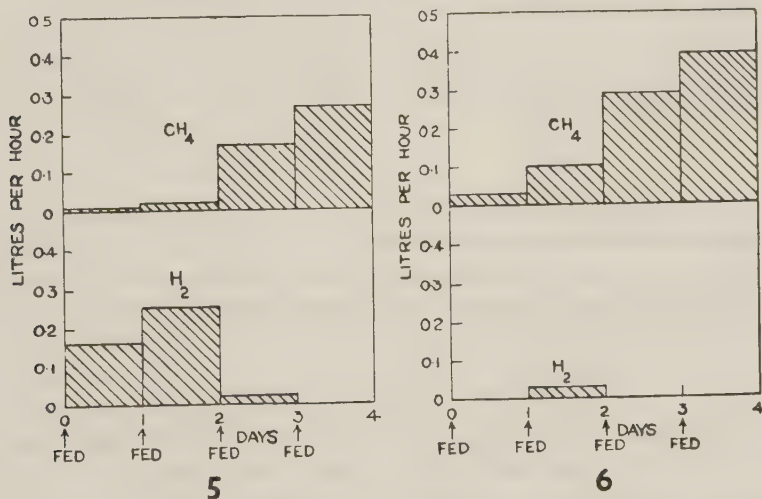


Fig. 5.—Production of methane and hydrogen by a sheep fed for 4 days after the rumen had been emptied.

Fig. 6.—Production of methane and hydrogen by a sheep fed for 4 days after 15 days fasting. A drench of rumen fluid from a regularly fed animal administered each day immediately prior to feeding.

siderable amounts of hydrogen (0.18-0.24 litre per hour) in the first two days and its subsequent replacement by methane. In the three experiments the maximum hydrogen production varied from 0.14 to 0.24 litre per hour.

The composition of gases in the rumen of an animal which had been treated similarly was examined in three experiments by the direct sampling method. The results from one of these experiments are shown in Figure 7. In this animal

almost 50 per cent. of the rumen gas, one day after feeding, consisted of hydrogen. By the end of the second day the hydrogen had largely been replaced by methane. The maximum proportions of hydrogen in the atmosphere above the rumen contents in these three experiments were 49 per cent., 22 per cent., and 57 per cent. respectively, the other gases at these peaks being methane 3 per cent., 1 per cent., and 2 per cent., carbon dioxide 35 per cent., 59 per cent., and 34 per cent., and nitrogen 13 per cent., 18 per cent., and 7 per cent.

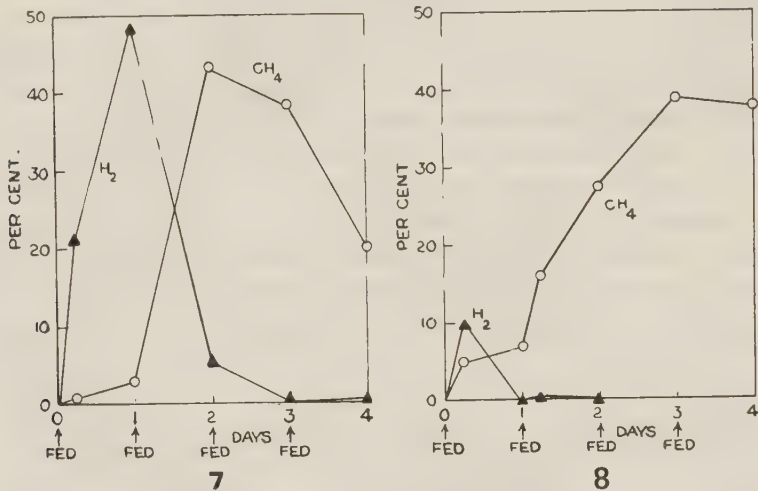


Fig. 7.—Percentage of methane and hydrogen in rumen gas withdrawn from a sheep fed for 4 days after the rumen had been emptied.

Fig. 8.—Percentage of methane and hydrogen in rumen gas withdrawn from a sheep fed for 4 days after the rumen had been emptied. A drench of rumen fluid from a regularly fed animal administered prior to the first feeding.

(d) Inhibition of Hydrogen Production

The appearance of free hydrogen in the gases evolved from the rumen contents when the conditions have been disturbed by prolonged fasting and its disappearance as methane production is resumed suggest that at least two organisms are involved in the production of methane in the rumen, one which produces hydrogen and another which reduces carbon dioxide to methane by means of the hydrogen so formed. The methane-producing organisms (cf. Barker 1936) are known to be non-spore forming, slow in development, prone to changes in environment, and especially sensitive to oxygen tension. They might be expected, therefore, to be greatly reduced in number in the rumen after the animal has fasted for a long period because the *milieu* of the rumen contents under such conditions is manifestly changed and the atmosphere above the contents altered greatly in composition. Similar changes in the environment were achieved simply by washing out the rumen contents through a fistula.

In order to illustrate further the processes involved, two sheep were fasted for 15 days and then fed in the respiration chamber. Immediately preceding the first and each subsequent feeding they were drenched with 150 ml. of rumen fluid withdrawn from a regularly fed sheep.

The animals used in the experiment were selected because previously on each occasion when they had been tested after long fasting periods hydrogen was produced at a rate of 0.2-0.5 litre per hour (Section (b)). Results from these two experiments were similar and one set of data is plotted in Figure 6. Only on one day was free hydrogen detected and then only in minute amount; methane production returned to normal values in 4 days.

Inhibition of hydrogen formation by inoculating with rumen fluid was also shown in a sheep in which the rumen had been emptied before feeding. In this case the direct sampling procedure was employed. Three experiments were made in which the rumen was emptied as previously described. In each case a single drench of approximately 150 ml. of rumen fluid was given immediately before the first feeding. The maximum concentrations of free hydrogen observed in these three experiments were 10 per cent., 12 per cent., and 2.5 per cent. respectively. These amounts are in marked contrast with the concentrations of 49 per cent., 22 per cent., and 57 per cent. which had previously been obtained from the same animal (Section (c)). The data from one of these experiments are shown in Figure 8.

IV. DISCUSSION

The production of methane in sheep after normal feeding was observed to follow the same trend as that found by Washburn and Brody (1937) to occur in cattle, but while these authors and other earlier writers (Tappeiner 1882; Markoff 1911; Zuntz *et al.* 1913) found small amounts of hydrogen when the analyses were made by volumetric methods, this gas was never detected in the present studies by the use of either gravimetric or volumetric methods. There are, however, conditions under which hydrogen does appear in the rumen; these are not encountered under normal feeding conditions.

It is generally agreed (cf. Barker 1936) that formation of methane by the fermentation of polysaccharides is due to the reduction of carbon dioxide either by hydrogen or by a variety of compounds that can act as hydrogen donors. The breakdown of polysaccharides under conditions where the methane-producing organisms are unlikely to be present, results in the formation of hydrogen (cf. Waksman 1932). It would appear, therefore, that formation of methane in the rumen is consistent with the view that at least two types of organism contribute to its formation. In the experiments described above, when conditions were such as to destroy or remove methane-producing organisms, free hydrogen was evolved initially, and it was demonstrated that its evolution could be inhibited by the addition of rumen fluid from a normally fed animal.

V. ACKNOWLEDGMENTS

The work described in this paper was carried out as part of the research programme of the Division of Biochemistry and General Nutrition, C.S.I.R.

The author is indebted to his colleagues, Messrs. F. V. Gray and E. W. L. Lines for valuable advice, and Mr. I. G. Jarrett for the establishment of fistulæ in the animals used. Particular acknowledgment is made to Mr. H. R. Marston, Chief of the Division of Biochemistry and General Nutrition, C.S.I.R., for his interest throughout the experiments and for his criticism and help in the preparation of the manuscript.

VI. REFERENCES

- BAILEY, C. V. (1921).—*J. Lab. Clin. Med.* 6: 667.
BARKER, H. S. (1936).—*Arch. Mikrobiol.* 7: 404.
COLE, H. H., *et al.* (1945).—*J. Anim. Sci.* 4: 183.
LINES, E. W. L. (1938).—*J. Agric. Sci.* 28: 663.
LUGG, J. W. H. (1938).—*Ibid.* 28: 688.
MARKOFF, J. (1911).—*Biochem. Z.* 34: 211.
SHEPHERD, M. (1931).—*J. Res. Nat. Bur. Stand.* 6: 121.
TAPPEINER, H. (1882).—*Ber. dtsh. chem. Ges.* 15: 999.
WAKSMAN, S. A. (1932).—"Principles of Soil Microbiology," 2nd Ed. (Williams and Wilkins Co.).
WASHBURN, L. E., and BRODY, S. (1937).—*Missouri Agric. Exp. Sta. Res. Bull.* No. 263.
ZUNTZ, N. R., HEIDE, R. v.d., and KLEIN, W. (1913).—*Landw. Vers. Sta.* 79-80: 781.

THE ESTIMATION OF CYTOCHROME C OXIDASE IN ANIMAL TISSUES

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Summary

A method for the precise estimation of the cytochrome oxidase in animal tissues is described.

The addition of 1-phenylsemicarbazide to the system *in vitro* containing hydroquinone as a substrate prevents the accumulation of inhibitory reaction products. Semicarbazide which was used formerly for this purpose is unsuitable since a gas, probably nitrogen, is formed when it reacts with the oxidation products of hydroquinone.

The effect of the phosphate buffer, and of other salts, upon the activity of the enzyme is described. It is important, for an estimation of cytochrome oxidase, that not only should the cytochrome concentration be adequate, but also the concentrations of buffer and other electrolytes should be controlled.

I. INTRODUCTION

Methods for the estimation of cytochrome c oxidase are dependent either upon the change which occurs in the absorption spectrum of cytochrome c when it is oxidized by cytochrome oxidase (Altschul, Abrams, and Hogness 1939; Albaum, Tepperman, and Bodansky 1946*a*, 1946*b*), or upon the absorption of gaseous oxygen by a system which consists essentially of a preparation of cytochrome oxidase in the presence of a large excess of reduced cytochrome c. In either case, it is the rate of oxidation of reduced cytochrome c which is estimated; in the former by spectrophotometric measurement of the rate of change in light transmission at two different wavelengths, and in the latter by manometric estimation of the rate of oxygen uptake (Keilin and Hartree 1938; Stotz 1939; Schneider and Potter 1943). The rate of oxidation of the reduced cytochrome c is proportional, under certain conditions, to the amount of cytochrome oxidase present, and so can be used as a measure of the activity of cytochrome oxidase itself.

For the manometric estimation of the rate of oxygen uptake cytochrome oxidase must be present in quantities small enough to limit the reaction, while cytochrome c should be in considerable excess and maintained in the reduced form by such readily oxidizable compounds as *p*-phenylenediamine, hydroquinone, or sodium *l*-ascorbate (Keilin and Hartree 1938; Stotz 1939; Schneider and Potter 1943).

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Some difficulties were experienced in the early use of hydroquinone for this purpose as the reaction products resulting from the oxidation of this compound rapidly decreased the rate of O_2 uptake by the system. To circumvent this difficulty Schultze (1939) added semicarbazide which enabled a steady rate of O_2 uptake to be maintained for 20 minutes. The use of semicarbazide, however, introduces other difficulties (Schneider and Potter 1943) since the net O_2 uptakes are always lower than those observed when sodium ascorbate replaces the hydroquinone as substrate. It became evident during an examination of the cytochrome c-cytochrome oxidase system in this laboratory that the apparent lowered uptakes were due to the simultaneous evolution of a gaseous reaction product. This disadvantage may be eliminated entirely if semicarbazide is replaced by 1-phenylsemicarbazide, and with the use of the latter it became possible to determine in greater detail the optimum conditions under which the cytochrome system functions *in vitro*, and so to develop a method for the estimation of the cytochrome c oxidase in animal tissues which might provide a useful alternative, in certain circumstances, to the method evolved by Schneider and Potter (1943).

II. GENERAL EXPERIMENTAL PROCEDURE

(a) *Apparatus and Reagents*

All manometric determinations of O_2 uptake were conducted with Warburg apparatus equipped with conical 15 ml. reaction vessels. About 100 or more oscillations per minute were found to be adequate to ensure that the amount of oxygen available to the oxidase was not limiting. When KOH solution or KOH-soaked papers were used in the centre well, the rim and walls of the centre well were greased with vaseline to prevent the KOH solution from seeping over into the reaction chamber. Lanoline was rejected for this purpose as some inhibition of O_2 uptake had been observed when the liquid phase came into contact with the lanoline.

Glass distilled water was used throughout. All reactions were carried out at 37°C.

Tissue Suspensions.—These were prepared by finely mincing the tissues with scissors and then dispersing them in glass distilled water by means of a glass homogeniser of the type described by Potter and Elvehjem (1936). This treatment was continued for approximately one minute, after which the suspension was diluted to the required volume and passed through fine gossamer veiling with a 0.2 mm. mesh. A thin pad of glass wool may be substituted for gossamer veiling without altering the activity of the suspension. For convenience, the dilutions were made so that the O_2 uptake for the greatest quantity of suspension likely to be used in a reaction vessel would not exceed 40 μ l. in five minutes. The following dilutions were found suitable: rat liver, 1 g. wet weight in 40 ml.; whole rat brain, 1 g. in 30 ml.; sheep liver and brain (grey matter) 1 g. in 20 ml.; sheep kidney cortex, 1 g. in 40 ml.

0.2M Hydroquinone.—A solution of pure benzohydroquinone in water was prepared each day.

0.1M 1-Phenylsemicarbazide.—The solid was dissolved in hot water and the solution kept at 37°C. in the water bath.

0.12M Sodium Ascorbate.—This was prepared each day by dissolving 105.6 mg. ascorbic acid in 3 ml. water, neutralizing to pH 7 with normal NaOH, and making up to a final volume of 5 ml. immediately before use. This provided an alternative substrate for comparison with hydroquinone.

Cytochrome C.—This was prepared from ox-heart muscle by the method of Keilin and Hartree (1937). The final dialysis was carried out against distilled water. Although some cytochrome c is lost by diffusion through the cellophane sac by this procedure, this disadvantage is offset by the lowered content of electrolytes in the final product. More recent preparations of cytochrome c were dialysed in "Visking High Stretch" sausage casings (size $1\frac{7}{8}$ in. by 20 in.). No cytochrome c was lost during dialysis when these were used.

The cytochrome c solution was removed from the sac, filtered, boiled and filtered again through No. 1 Whatman paper. This procedure removes interfering materials without impairing the oxygen-carrying capacity of the cytochrome c. The solution of cytochrome c may be used in this condition or it may be dried in a vacuum desiccator to a dark red solid which may be stored.

0.4M Sodium Phosphate Buffer.—This was prepared from 0.4M Na_2HPO_4 and 0.4M NaH_2PO_4 . A predetermined titration curve enabled a buffer of any desired pH to be prepared. The potassium salts could be substituted for the sodium phosphates. In our experience this change had little influence on the system at any given pH.

(b) Outline of Method

The estimation of cytochrome oxidase entails the measurement of the rate of oxygen uptake of a system limited only by the oxidase content—reduced cytochrome c and oxygen being in large excess. Cytochrome c may be kept in the reduced state by the use of a suitable and easily oxidizable substrate—in this case hydroquinone. It is necessary to remove reaction products which influence the oxygen uptake. 1-Phenylsemicarbazide was found adequate for this purpose. The concentration of electrolytes was found to have a definite effect upon the cytochrome oxidase activity. It is necessary, therefore, to establish by preliminary experiments the quantities of buffer and of cytochrome c which must be used.

When the optimal quantities of buffer and of cytochrome c which are to be used have been ascertained (*vide infra*) the following procedure may be adopted. In each of six Warburg vessels are placed:

- (1) The optimal amount of 0.4M phosphate buffer, pH 7.1.
- (2) The correct amounts respectively of cytochrome c and of 0.2M hydroquinone—the latter in the side-arm ready to be tipped into the reaction chambers after the vessels have equilibrated to 37°C.

- (3) The same amount of phenylsemicarbazide solution in each.
- (4) Five different quantities of tissue suspension, and one amount of tissue suspension which has been boiled to inactivate the oxidase. This permits the measurement of the O_2 uptakes for five separate systems containing different amounts of cytochrome oxidase and of one system containing inactivated oxidase.
- (5) Sufficient water to make all the systems up to the same total liquid volume.

After an equilibration period of five to ten minutes, the manometers are levelled and the taps closed; the contents of the side-arms are then tipped into the reaction chambers and the time is noted. Two minutes later the first readings are taken. These are regarded as the readings for zero time; the measurement of rates of O_2 uptake begins at this moment. In this way, allowance is made in the first two minutes for all inequalities due to the different times of tipping and return to the bath or to other initial variables. Thereafter, four consecutive readings at five-minute intervals are taken. From these readings the total O_2 uptakes for each of the six Warburg vessels at 5, 10, 15, or 20 minutes after zero time may be calculated. These values for cumulative uptakes are plotted against the time (Fig. 1). Six straight lines corresponding to the O_2 uptakes result which, when extrapolated, allow the total O_2 uptake after 30 minutes to be estimated for each of the systems. Suitable corrections may be applied to any of these lines which does not pass through the point of zero uptake at zero time by drawing through this point a straight line parallel to the line in question. This will cut the 30-minute ordinate at the correct value, e.g. "autoxidation blank" (Fig. 1). The 30-minute O_2 uptake values determined in this way are then plotted against the concentrations of tissue suspension. The result is a straight line which indicates the direct proportionality between oxygen uptake and ml. of suspension, i.e., amounts of cytochrome oxidase (Fig. 2). It has been customary in this laboratory to regard the intercept of this line on the "y" axis as a measure of the autoxidation of the substrate—in this case of the hydroquinone. Parallel experiments have shown that the autoxidation of hydroquinone under similar conditions is unaltered by the addition of cytochrome, or of tissue suspension. Allowance is made for this autoxidation when assessing the net O_2 uptake by the system containing the known amount of tissue suspension. The O_2 uptake may be expressed in terms of the dry matter or the nitrogen content of the tissue suspension. The conventional expression " QO_2 " is used in this laboratory to mean " μ l. of O_2 uptake/hr./mg. dry matter" in the preparation containing the oxidase. This method has been employed by us for several years and was used when investigating the enzymic destruction of ascorbic acid (Marston, Quinlan-Watson, and Dewey 1943).

Schneider and Potter (1943) have employed a graphical treatment for their results from which they assess the rate of oxidation of the substrate in a manner similar to that described above.

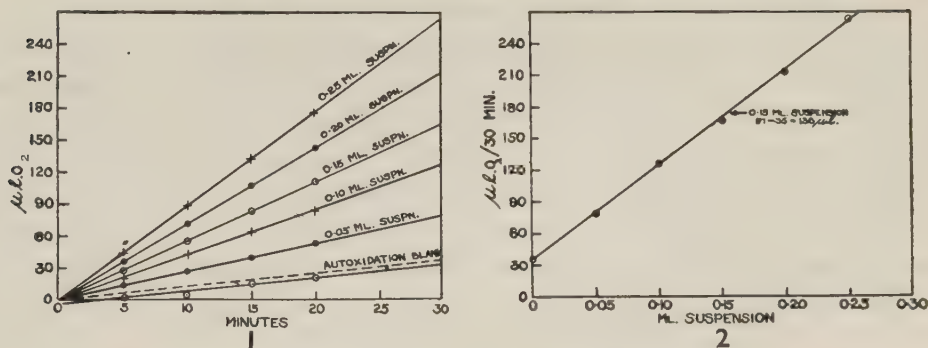


Fig. 1.—Oxygen uptakes for different amounts of tissue suspension; the contents of the Warburg vessels were 0.5 ml. of 0.4M phosphate buffer (pH 7.1), 0.6 ml. of cytochrome c solution,* 0.25 ml. of 0.1M 1-phenylsemicarbazide, 0.25 ml. of 0.2M hydroquinone in the side-arms, and amounts of tissue homogenate varying from 0.05 to 0.25 ml. The autoxidation blank contained 0.25 ml. of boiled suspension. Water was added to each system to make the total liquid volume = 2.5 ml.

*The strength of this cytochrome c solution, which was found adequate in amount for oxidase assay was not determined, but from later work it is probable that its concentration was of the order of 0.8-1.0mM.

Fig. 2.—Cytochrome oxidase assay curve. Time = 30 min. The values for oxygen uptakes have been obtained from the 30-min. ordinate in Figure 1.

III. CONDITIONS GOVERNING THE PROPER FUNCTIONING OF THE SYSTEM

(a) *The Hydrogen Donator Substrate*

(i) *Hydroquinone*

In systems containing hydroquinone, the rate of oxygen uptake falls off rapidly owing to the inactivation of the enzyme by the reaction products. To circumvent this, some authors sacrifice precision by assessing the oxidase activity from the figures of O_2 uptake for the first five minutes. More precise results are obtainable only if the rate of the reaction is estimated by measuring the velocity of the O_2 uptake over a period of about 20 minutes.

(ii) *Hydroquinone plus Semicarbazide*

Schultze (1939) introduced semicarbazide into the system to maintain the activity of the enzyme. Although the oxidase retains its activity for a longer period under such circumstances, it became clear, early in the present study, that the initial O_2 uptake, in the presence of semicarbazide, was not as great as when reacting systems free from semicarbazide were employed (Fig. 3). Schneider and Potter (1943) have since reported a similar experience, and because of this, prefer to discard hydroquinone in favour of *l*-ascorbic acid as a substrate.

Investigation of the cause of the lowered O_2 uptake in systems containing semicarbazide revealed that over the pH range of activity of the oxidase, quinone and semicarbazide do not react directly to form the semicarbazone;

the combination results in the evolution of a gas, probably of nitrogen. In a search for efficient means of overcoming the depressing action exerted by oxidation products of hydroquinone, the behaviour of dimethylamine, phenylurea, thiosemicarbazide, 1-phenylsemicarbazide, phenylthiosemicarbazide, and 4-phenylsemicarbazide was studied. The first two compounds mentioned either failed to combine with quinone under the conditions of the assay or the complex formed partially inactivated the enzyme; 4-phenylsemicarbazide, phenylthiosemicarbazide, and thiosemicarbazide, like semicarbazide, reacted with gaseous end products.

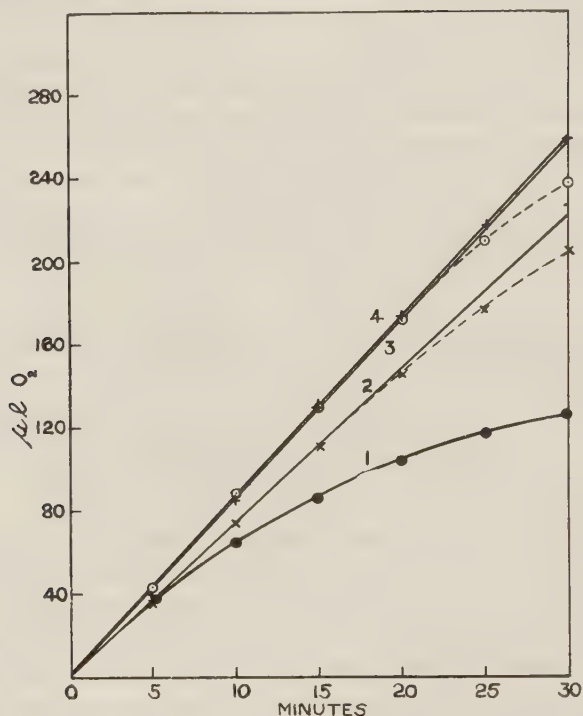


Fig. 3.—The oxygen uptakes of the cytochrome system with different substrates. The substrates were (1) hydroquinone alone, (2) hydroquinone and semicarbazide, (3) hydroquinone and 1-phenylsemicarbazide, (4) sodium ascorbate. When sodium ascorbate was used 0.2 ml. of 20 per cent. KOH and fluted filter-paper strips were placed in the centre well. 0.2 ml. of 1/30 tissue suspension in all.

(iii) 1-Phenylsemicarbazide

The inactivating effect of quinone was found to be circumvented by 1-phenylsemicarbazide which reacts without the evolution of gas. The differences between the QO_2 's obtained when either *l*-ascorbic acid or hydroquinone plus 1-phenylsemicarbazide were employed as substrates in systems containing emulsions of rat brain were small (Table 1).

TABLE 1

Suspension No.	Total O ₂ Uptake* minus Autoxidation = Net O ₂ Uptake* Hydroquinone + 1-phenylsemicarbazide (μ l. O ₂ /30 min.)	Sodium Ascorbate (μ l. O ₂ /30 min.)	Difference (%)
1	162 — 48 = 114	178 — 64 = 114	0
2	146 — 39 = 107	163 — 48 = 115	+8
3	174 — 44 = 130	210 — 85 = 125	—3
4	170 — 41 = 129	232 — 47 = 185	+3.9
5	175 — 44 = 131 } 179 — 44 = 135 }	204 — 52 = 152 } 207 — 52 = 155 }	+15
6	169 — 37 = 132	198 — 52 = 146 } 199 — 54 = 145 }	+11
7	159 — 33 = 126	180 — 47 = 133	+6
8	183 — 42 = 141	205 — 54 = 151	+7

*In 0.15 ml. of 1/30 suspension of rat brain.

(iv) *Changes in the Concentration of Hydroquinone*

The amounts of 0.2M hydroquinone and 0.1M 1-phenylsemicarbazide which will establish optimal conditions for the proper functioning of the system may be varied over considerable limits. Under the conditions employed, hydroquinone did not impose any limitation until final concentrations in the systems lower than 0.01M were reached: it showed signs of toxicity to the system, however, at final concentrations greater than 0.045M. Between these limits, the only effect of a change in hydroquinone concentration was a change in the rate of autoxidation implied by the intercept on the "y" axis—the slope of the straight line, which was a measure of the oxidase, remained constant (Fig. 4).

(v) *Changes in the Concentration of 1-Phenylsemicarbazide*

Final concentrations below 0.005M were found to be insufficient to combine completely with the reaction products formed; concentrations above 0.045M were inhibitory to a moderate degree (about 20 per cent.). The oxidase activity was retained most efficiently by our concentrations of 0.01-0.02M (Fig. 6).

(vi) *Changes in the Oxygen Tension*

The only noticeable consequence of increasing the partial pressure of oxygen in the gaseous phase (from air to pure O₂) was a change in the autoxidation of the system. The line relating O₂ uptake to concentration of oxidase was merely translated; its slope remained constant (Fig. 5). In experiments with rat liver, a slight depression in the slope of the curve was observed which suggested that O₂ might have been slightly inhibitory to the system at high partial pressures (cf. Stadie and Haugaard 1945; Dickens 1946), but in view of the lability of the enzyme in liver preparations no significance was attached to this observation.

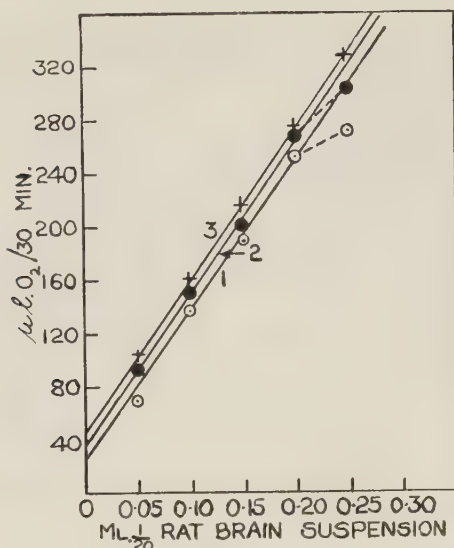


Fig. 4.—Cytochrome oxidase assay curves with different concentrations of substrate. 0.6 ml. of 0.1M l-phenylsemicarbazide in each and 0.3 ml. of hydroquinone solution as substrate. Strengths of the hydroquinone solutions and final concentrations in the Warburg vessel were (1) 0.1M, 0.012M; (2) 0.2M, 0.024M; (3) 0.4M, 0.048M.

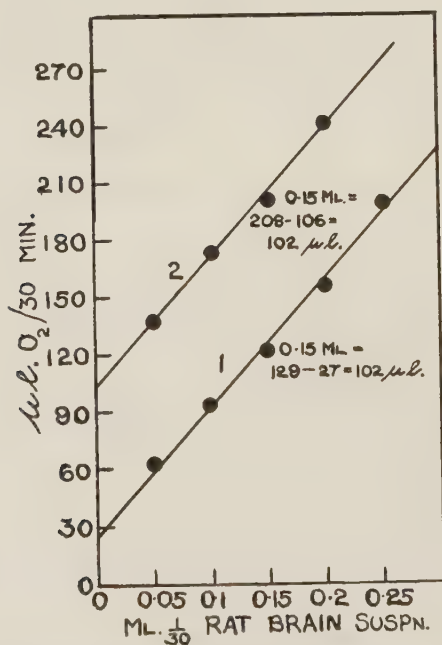


Fig. 5.—The effect of oxygen upon the cytochrome oxidase assay curve (1) in air, (2) in oxygen.

Although it appears from Table 1 that *l*-ascorbic acid is slightly superior as a substrate to hydroquinone plus 1-phenylsemicarbazide, the method provides a useful and independent check upon QO_2 's obtained with *l*-ascorbic acid as a

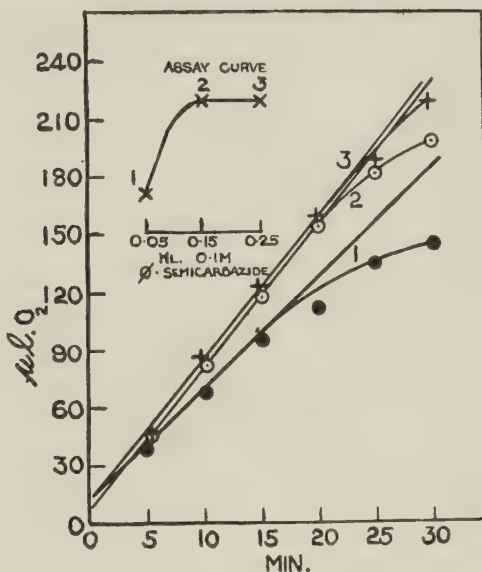


Fig. 6.—The effect of changes in the concentration of 1-phenylsemicarbazide upon the cytochrome system *in vitro*. In the Warburg vessels were: 0.45 ml. of 0.4M phosphate buffer pH 7.1, 1.0 ml. of cytochrome c solution, 0.15 ml. of 1/30 rat brain suspension, 0.25 ml. of 0.2M hydroquinone in the side-arm, and amounts of 0.1M 1-phenylsemicarbazide equal to (1) 0.05 ml., (2) 0.15 ml., (3) 0.25 ml. The total volume of 2.5 ml. was made up with water.

substrate, and since no CO_2 is evolved, the employment of folded paper strips soaked in KOH solution is unnecessary. This enables experiments with cyanides and similar substances to be performed without introducing errors due to the complication, exposed by Riggs (1945), which is caused by the cyanide distilling over to a KOH solution in the centre well. More direct interference from ascorbic oxidase is circumvented.

(b) Preparation of the Tissue Suspension

The stability of cytochrome oxidase was investigated in tissues subjected to various treatments after their removal from the animal. Freezing by immersion of a beaker containing the tissue in a mixture of ice and salt did not appear harmful to the enzyme, but when the tissue was removed from the animal and dropped into solid CO_2 there was a marked decrease in the cytochrome oxidase activity of the suspension subsequently made from it. An inhibition of between 10-30 per cent. was noticed in sheep liver and about 60 per cent. in rat liver. Enzyme activity was apparently preserved more efficiently in intact tissues than

in suspensions. It is preferable, therefore, if the tissue has to remain some time before being examined for its cytochrome oxidase content, that it should be kept intact in the refrigerator, i.e. at a temperature of 0°-4°C. The enzyme activity is unchanged for several hours under these conditions.

The directions for preparing a suspension of any soft tissue have already been given. Suspensions of rat liver, sheep cerebral cortex, sheep kidney cortex, sheep liver, rabbit liver or brain, and guinea pig liver prepared in this way were not stable for any considerable time. The decrease in oxidase activity was more rapid at room temperatures (15°-25°C.) than at 4°C. (rat liver suspension 20-30 per cent. at room temperature, under 10 per cent. at 4°C. in 4-5 hours; 20-40 per cent. in 24 hours at 4°C.).

On the other hand, suspensions prepared from the whole cerebral hemispheres of rats showed no decrease in activity after 24 hours, or in some cases 48 hours, storage at 0°-4°C. For this reason, rat brain suspensions were used in the majority of the experiments reported in this paper, as a number of experiments could be made upon the same suspension.

Care must be taken to ensure that a minimum of inactivation of the enzyme occurs during the preparation of the suspension. Some authors (Albaum, Tepperman, and Bodansky 1946*a*, 1946*b*) have used the Waring Blender to effect a dispersal of the tissue. We have found, however, that this procedure rapidly inactivates the enzyme (Fig. 7). An inhibition of more than 30 per cent. in the cytochrome oxidase activity of guinea pig liver was observed by treatment for one minute at room temperature in the Waring Blender at approximately 12,000 r.p.m. with no significant rise in temperature of the main bulk of the liquid.

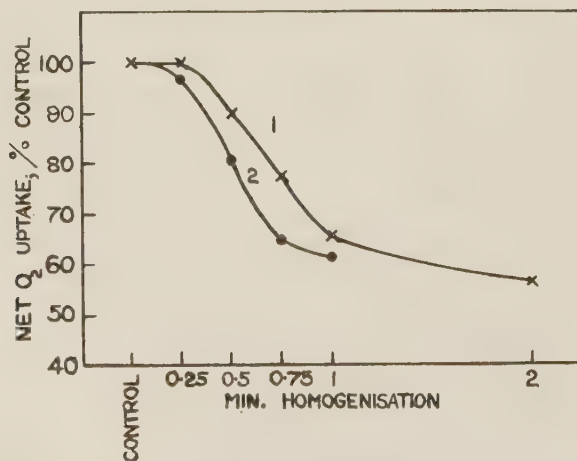


Fig. 7.—Effect of the Waring Blender on tissue suspension. 0.2 ml. of 1/20 guinea pig liver homogenate in all Warburg vessels. Rotor speeds without any load: (1) low speed = 11,000 r.p.m. (2) high speed = 16,000 r.p.m.

Less destruction of the enzyme was observed when the glass homogeniser* was used. When the rotor was moving at nearly constant speed (c. 1,500-2,000 r.p.m.), the oxidase of guinea pig liver was gradually inactivated as the time of treatment increased (Fig. 8), although the amount of inactivation which occurred during the first minute, in contrast with the Waring Blendor, was negligible.

If the minced tissue suspended in water was treated for a fixed time, the degree of inactivation of the oxidase depended upon the speed of the rotor (Fig. 9). Cooling the apparatus in ice or displacing the air by nitrogen had no effect.

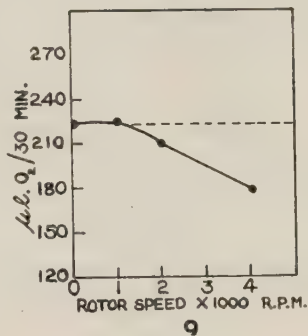
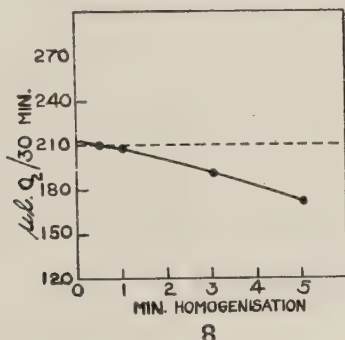


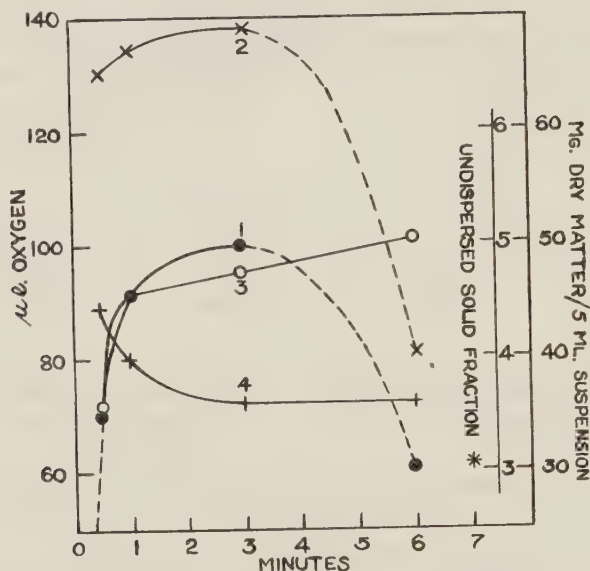
Fig. 8.—Tissue suspension treated for varying times in the glass homogeniser. 0.2 ml. of 1/20 guinea pig liver homogenate in all Warburg vessels. Rotor speed 1,000-2,000 r.p.m., rotor diameter 16 mm.

Fig. 9.—Tissue suspension homogenised at varying speeds in the glass homogeniser. Time of homogenisation in each case = 4 min. 0.20 ml. of 1/20 guinea pig liver homogenate in all Warburg vessels.

For maximum recovery of the cytochrome oxidase activity in the tissue suspension, both the speed of the rotor and the time of treatment should be reduced to the minimum which will ensure dispersion of the tissue. For relatively slow speeds (c. 1,000 r.p.m.) the total O_2 uptakes for unit volumes of suspension prepared from rat liver were found to increase with the time of treatment up to a maximum, after which partial inactivation became apparent. The amounts of dry matter in fine dispersion increased, while the larger particles, which were left on the gossamer sieve after straining the suspension, steadily decreased (Fig. 10). Thus the amount of tissue dispersed into fine suspension, which increased steadily with the time of treatment, was responsible for the observed increase in the O_2 uptake per unit volume of the suspension. The change in the QO_2 's (total O_2 uptakes/hr./mg. dry matter) was small, however, provided the time of treatment was not extended until partial inactivation became apparent.

*The homogeniser differed in minor respects from that described by Potter and Elvehjem (1936). It was made by joining a glass spindle to the inner of two closely fitting pyrex test tubes, the upper portion of the outer tube being enlarged to form a reservoir for fluid above the close-fitting rotor. The smooth rotor was 1.6 cm. diam. by 9 cm.

This would suggest that, if only a portion of the tissue is completely dispersed, and the undispersed portion removed, the QO_2 obtained for this suspension will be a very close measure of the cytochrome oxidase of the original tissue. It is desirable, however, to disperse as much of the original tissue as possible within the experimental limits set by the behaviour of the homogeniser as by this means a better sample is obtained.



*Expressed as $\frac{\text{mg. dry matter in solid or gossamer veiling}}{\text{mg. wet wt. of tissue before treatment}} \times 100.$

Fig. 10.—Rat liver treated for varying times in the glass homogeniser. Rotor speed $<1,000$ r.p.m. Portions of same liver made to 1/40 suspension in each determination. Oxygen uptakes for 0.15 ml. suspension estimated from graphs similar to those in Figures 1 and 2. Curves: (1) O_2 uptake/30 min./0.15 ml. of suspension; (2) QO_2 (μl. O_2 /hr./mg. dry matter in suspension); (3) dry matter in 5 ml. of suspension; (4) undispersed solid particles left on gossamer sieve.

The tissue should be finely minced with scissors before transferring to the homogeniser, and the rotor manipulated by hand to effect disruption of the tissue before homogenisation at 1,500-2,000 r.p.m. is attempted. The latter treatment should not exceed 1 minute. The suspension is diluted with water to a known volume and strained.

Experiments conducted with rabbit brain indicated that suspensions in distilled water were much more active than those prepared in Ringer-phosphate solutions. This might in part be due to greater plasmolysis in the water. Schneider and Potter (1943) have convincingly demonstrated that only the components of those cells which are disrupted contribute to the cytochrome oxidase activity *in vitro*. These authors investigated the proportion of unbroken

cells by a method involving an estimation of the succinoxidase activity of the suspension. We employed this method and found less than 10 per cent. of unbroken cells in suspensions prepared in the above manner.

(c) *Cytochrome C*

The dark red solution of cytochrome c which remains after the preparation has been dialysed and filtered may be used for the estimation. It may be stored in the refrigerator for a fortnight, but it is preferable to boil the solution and filter it again before use. This filtrate may then be dried in the vacuum desiccator. The dry powder which results may be stored for several months. We have found solutions containing between 7 and 15 mg. of the powder per ml. (approx. $0.5\text{--}1.0 \times 10^{-3}$ molar as determined by the method of Rosenthal and Drabkin (1943)) to be suitable for oxidase estimation.

(i) *Cytochrome C Assay*

Each new preparation of cytochrome c should be assayed to ensure that the concentration in the system is not a limiting factor. The procedure for this assay is as follows. In each of six Warburg vessels are placed:

- (1) 0.5 ml. of 0.4M sodium phosphate buffer, pH 7.1. The final total liquid volume is 2.5 ml.; consequently the buffer concentration becomes 0.08M and the pH 7.3.
- (2) A quantity of tissue suspension which can be expected to contain more cytochrome oxidase than the greatest amount used in an oxidase assay; 0.25 ml. of a suspension containing 1 g. of rat brain in 30 ml. is usually sufficient.
- (3) 0.25 ml. of 0.1M 1-phenylsemicarbazide.
- (4) 0.25 ml. of 0.2M hydroquinone in the side-arm.
- (5) Different amounts of (approx.) 0.5×10^{-3} M cytochrome c solution, ranging from 0.3 ml. to 1.2 ml.
- (6) Sufficient water to make the total liquid volume in all vessels 2.5 ml.

The buffer and water are added to the cytochrome c in the vessels before tissue suspension is added. This prevents the precipitate which forms when cytochrome c solution and tissue suspension are mixed (*vide infra*). The 1-phenylsemicarbazide solution and tissue suspension are added just before the vessels are placed in the water-bath.

The procedures of equilibration, tipping, reading, and the graphical treatment of the results are identical with those which have already been described above. Four 5-min. readings are taken after zero time, and the calculated O_2 uptake values for each system at the end of 30 min. can be obtained by extrapolation and plotted against the amount of cytochrome c (Fig. 11). From the figure it is evident that, for this particular amount of oxidase, quantities of cytochrome c less than that in 0.9 ml. of solution are definitely insufficient for

maximal O_2 uptakes by the oxidase. Quantities greater than this would be adequate for amounts of oxidase less than that in the 0.25 ml. of rat brain suspension used.

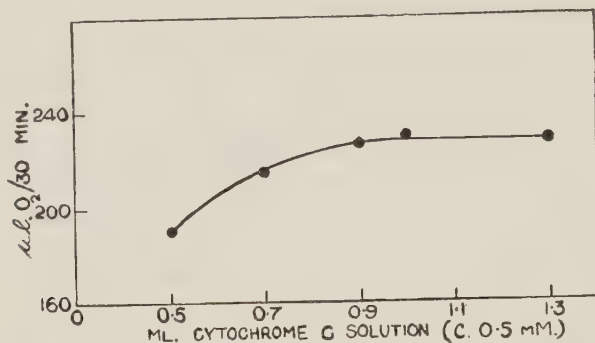


Fig. 11.—Cytochrome c assay, 0.25 ml. of 1/30 rat brain suspension used throughout.

(ii) *Insufficient Cytochrome C*

If insufficient cytochrome c is used for a cytochrome oxidase assay this may not be evident unless a cytochrome c assay has already been done. A straight line is still obtained when O_2 uptakes are plotted against amounts of tissue suspension, but its slope is much less than that of the line obtained when the O_2 uptakes are measured for the same amounts of tissue suspension in the presence of adequate cytochrome c (Fig. 12). The QO_2 obtained from the lower line in Figure 12 would be an erroneous one.

These results can be explained by assuming that cytochrome oxidase and cytochrome c reversibly combine to form a complex (Stotz, Altschul, and Hogness 1938) and that it is this complex which oxidizes hydroquinone. In other words, the O_2 uptake of the system must be proportional to the amount of complex present. In a typical cytochrome c assay, therefore, such as the one illustrated in Figure 11, the rising portion of the curve represents the increasing amount of complex formed by increasing the cytochrome c concentration, while the flattened, asymptotic portion of the curve represents the condition when the cytochrome c concentration is so great that all the free cytochrome oxidase has combined to form the complex. It is only when the cytochrome c is in excess, i.e. when its concentration lies somewhere on the asymptotic portion of the curve, that a measurement of O_2 uptake will give an accurate measure of the cytochrome oxidase of the tissue, since under such circumstances all the oxidase is combined in the complex. If cytochrome c is limiting, a portion only of the oxidase will be in combination and it is this portion whose QO_2 is measured in the lower line in Figure 12. Since the tissue suspension cannot oxidize the hydrogen-donor substrate until a relatively enormous excess of cytochrome c, compared with the amount normally found in tissues, is added to the suspension, it seems that the cytochrome c originally mediating the transfer of hydrogen to the oxidase in the cell is dissociated completely from the oxidase when the

cellular contents are dispersed, and is thus rendered ineffective. Presumably the arrangement and spatial position of the tissue constituents in the cell enable the oxidase to function with the meagre amount of cytochrome c present, although it is noteworthy that the rate of oxidation by cytochrome oxidase in the cell, as measured by the overall respiratory rate, is only one-fiftieth to one-hundredth of the rate observed when extra cytochrome c and a substrate are added *in vitro*. Possibly the concentration of cytochrome c limits oxidation even *in vivo*.

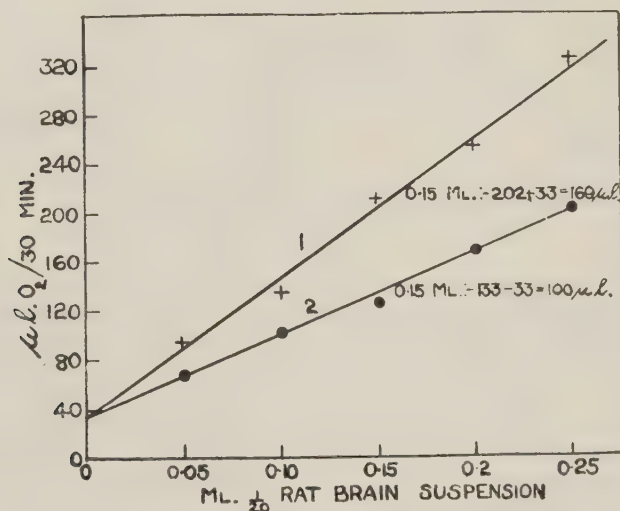


Fig. 12.—Cytochrome oxidase assay with insufficient cytochrome c. (1) Usual assay, cytochrome c in excess; (2) same assay, with one-half the amount of cytochrome c used in (1), i.e. cytochrome c limiting.

(iii) Boiled Cytochrome C Solution

Occasionally cytochrome c solution will begin to decompose if kept for longer than 10 days to 2 weeks without preservative; the solution then becomes less efficient in reducing cytochrome oxidase, and a lower apparent QO_2 will result (Fig. 13). The blank O_2 uptake value, as measured by the intercept on the “y” axis, was observed to increase under such circumstances.

A brown precipitate usually forms on boiling the cytochrome solution. This may be removed by filtration; the resulting filtrate has an increased ability to reduce cytochrome oxidase and it no longer displays any tendency to autoxidize (Fig. 14). The autoxidation of the whole system as indicated by the “y” intercept. (Fig. 14) was observed to decrease from 72 $\mu\text{l.}$ to a more customary figure of 47 $\mu\text{l.}$ (Figs. 13 and 14).

On occasions a fresh preparation of cytochrome c, after dialysis and filtering, tends to autoxidize. A brown precipitate usually forms when this solution is boiled. After removal of the precipitate it was observed that the filtrate no

longer tended to autoxidize and so such solutions are suitable for oxidase estimation. It has thus been customary in this laboratory to boil and filter a cytochrome c solution before it is used.

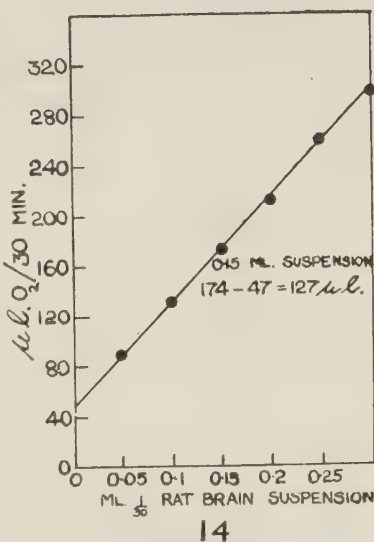
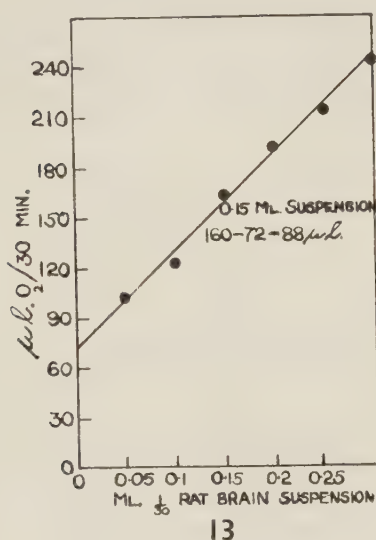


Fig. 13.—Cytochrome oxidase assay with old unboiled cytochrome c solution.

Fig. 14.—Cytochrome oxidase assay with boiled and filtered cytochrome c solution. The tissue suspension is the same as in Figure 12; cytochrome c solution the same except that it has been boiled and filtered.

(d) *The Influence of Electrolytes upon the System*

The oxygen uptake for any particular suspension was observed to vary with the concentration of the phosphate buffer (Fig. 15). The maximal values were usually achieved when the final phosphate concentration was between 0.072M and 0.088M. For this reason a final concentration of 0.08M phosphate is chosen when cytochrome c is assayed. The position of the peak of the curve relating O_2 uptake and sodium phosphate buffer concentration appeared to shift slightly within those limits of phosphate concentration when different preparations of cytochrome c were employed (Fig. 15). It is thus advisable to find the optimal buffer concentration for each preparation of cytochrome. The 30-min. O_2 uptakes of systems in which only the buffer concentrations are varied may be obtained by the procedures already described, and plotted against the quantities of buffer solution to give a curve similar to B in Figure 15. The peak indicates that 0.45 ml. of phosphate buffer is the amount to use with this preparation of cytochrome c.

When adequate cytochrome c was present, and the oxidase was limiting, the peak was observed to be unchanged in position irrespective of whether different tissue suspensions, or different amounts of the same suspension (Fig.

16) were used. When cytochrome c was limiting, however, the position of the peak was observed to shift along the phosphate concentration ordinate as the cytochrome concentration was increased (Fig. 17). If the total liquid volume was changed, it became necessary, in order to obtain maximal O_2 uptakes, to

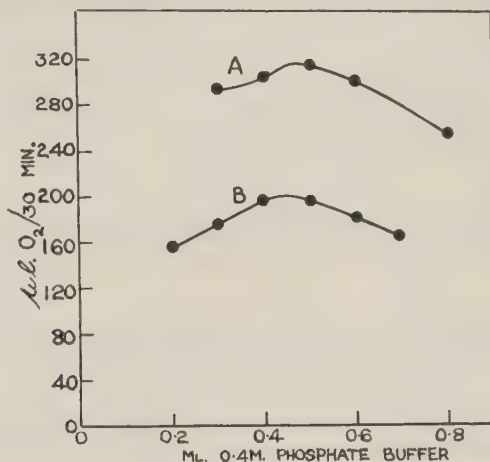


Fig. 15.—The variation in total oxygen uptake of the cytochrome system *in vitro* when the buffer concentration is altered. Curves A and B are for two different preparations of cytochrome c. 0.25 ml. of 1/30 rat brain suspension in all.

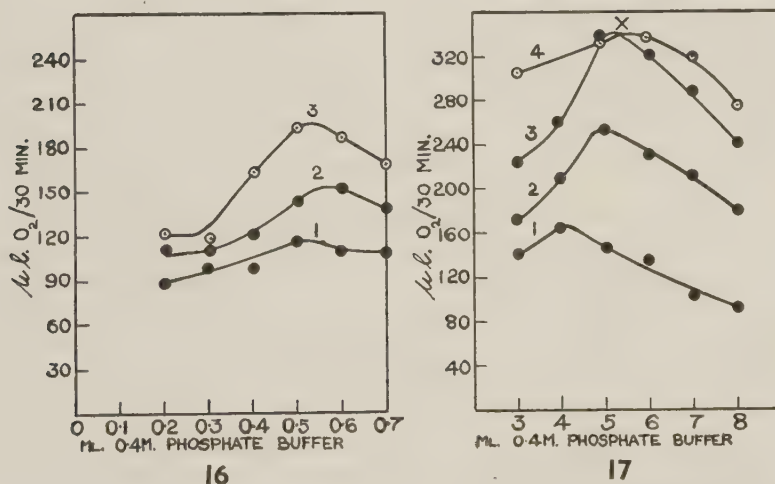


Fig. 16.—The variation due to a change in phosphate concentration in the total oxygen uptake of the cytochrome system *in vitro* for three different amounts of tissue suspension. Amounts of 1/30 rat brain suspension were: (1) 0.1 ml., (2) 0.2 ml., (3) 0.3 ml.

Fig. 17.—The variation due to change in phosphate concentration in the total oxygen uptake of the cytochrome system *in vitro* for different concentrations of cytochrome c; ml. of cytochrome c solution used: (1) 0.1 ml., (2) 0.3 ml., (3) 0.5 ml., (4) 0.6 ml. Cytochrome c was limiting in quantities below 0.55 ml. of solution.

increase the amount of 0.4M phosphate buffer so that the final concentration of phosphate remained the same (Figs. 18 and 19). If this was done the same QO_2 's were obtained for a tissue suspension even though the total volume had changed. The autoxidation—and hence the total O_2 uptake—was lowered if the volume was increased, as would be expected from the lower concentration of hydroquinone in the larger volume.

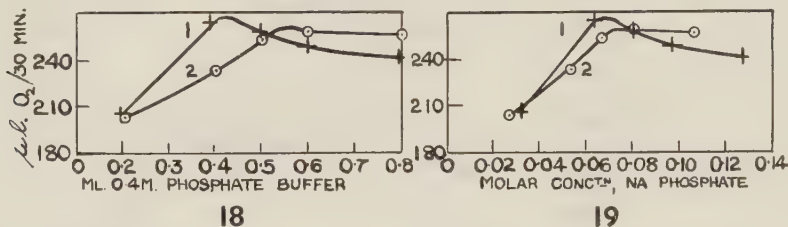


Fig. 18.—The variation in total oxygen uptake of the cytochrome system *in vitro* produced by increasing amounts of phosphate. In (1) the total liquid volume is 2.5 ml., in (2) 3 ml.

Fig. 19.—The data from Figure 18 plotted to show the relationship between total oxygen uptake and final molar concentration of sodium phosphate.

(i) The Influence of pH on the Buffer Concentration Effect

A dilution of 0.4M sodium phosphate buffer to 0.08M increases the pH from 7.1 to 7.3. The slight change in the pH (pH 7.26-7.32) due to variation in the concentration of the buffer (from 0.112M to 0.032M), however, is not the cause of this relation between buffer concentration and O_2 uptake of the oxidase-cytochrome system. The buffer concentration- O_2 uptake curves obtained at final observed reactions of pH 7.1, 7.3, and 7.36 were similar in their tendency to rise to a maximum value and then decline (Fig. 20).

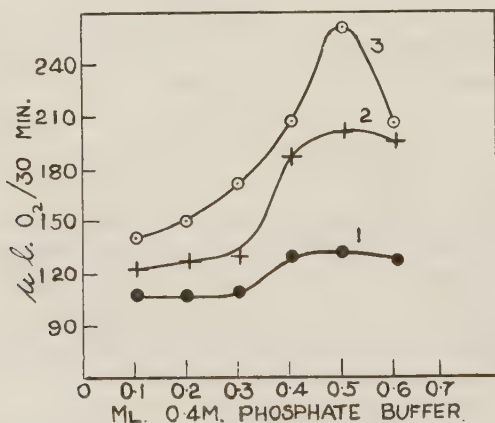


Fig. 20.—The effect of pH upon the variation produced in total oxygen uptakes by changes in the phosphate concentration; (1) pH 7.1, (2) pH 7.3, (3) pH 7.36.

(ii) *The Influence of a Large Excess of Cytochrome on the Buffer Concentration Effect*

It was thought that a large increase in the cytochrome c concentration might abolish the buffer concentration effect altogether. However, experiments in which a large excess of cytochrome was present in the system resulted in a buffer curve similar to that obtained when systems were studied in which the cytochrome c concentration was just adequate to ensure maximum O_2 uptake by the oxidase (Fig. 21).

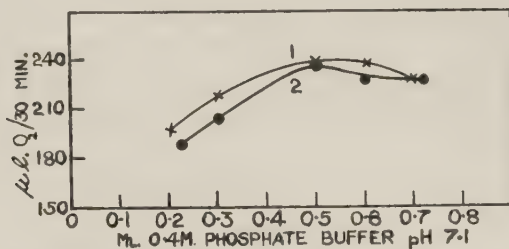


Fig. 21.—The variation produced in total oxygen uptake by changes in the phosphate concentration; the effect of large excess of cytochrome c. Final concentrations of cytochrome c in the Warburg vessel were: (1) c. 0.2mM, (2) 0.4 mM.

(iii) *The Influence of Sodium Ascorbate on the Buffer Concentration Effect*

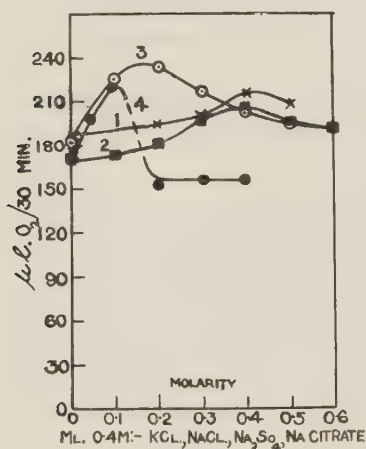
When hydroquinone substrate was replaced by sodium ascorbate this buffer concentration- O_2 uptake relationship could still be observed. The peak of the curve in the latter circumstances occurred at slightly lower concentrations of buffer. This shift in the peak might conceivably be due to the extra ions of sodium and ascorbate.

(iv) *Effect of Other Electrolytes*

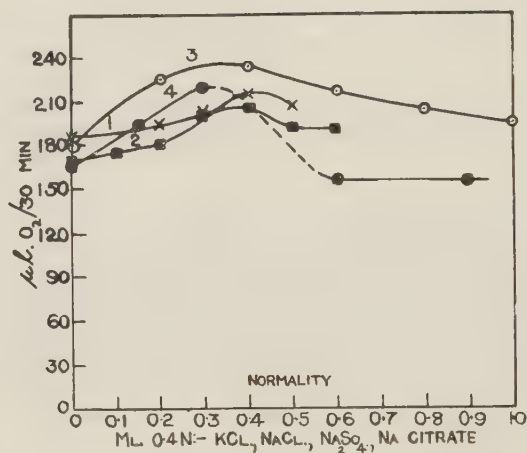
When cytochrome c, hydroquinone, 1-phenylsemicarbazide, and tissue suspension were all present in adequate and constant amount, and the concentration of phosphate buffer was kept at a level which would maintain a constant pH, but which was too low for maximal O_2 uptake by the system, the addition of other electrolytes stimulated the O_2 uptake. For example, if the buffer concentration was maintained at 0.032M (0.2 ml. of 0.4M phosphate in 2.5 ml.) and increasing amounts of NaCl (or KCl) were added to the systems, a curve of O_2 uptake was obtained which rose to a peak at a definite chloride concentration and thereafter declined as the chloride concentration increased. This resembled the phosphate curve, except that the peak occurred at a different molar concentration (Fig. 22).

If Na_2SO_4 were substituted for the chloride, a curve, which rose to a peak at a different molar concentration of electrolyte, was obtained. A similar effect was observed when sodium citrate was used, although once more the peak occurred at a different molar concentration (Fig. 22).

The peaks of the curves relating O_2 uptake and normality when NaCl, Na_2SO_4 , or sodium citrate were present, however, occurred in systems of approximately the same normality for each one of these salts (Fig. 23). These curves appeared to be in some way dependent upon the condition of the tissue suspension, since a number of suspensions produced the effects just described, while with other suspensions aberrant O_2 uptakes were observed when chloride or sulphate was varied. With some tissue suspensions variation in the electrolyte concentration had no apparent effect upon the system. The effect of buffer concentration, however, was invariably observed. Although the general shape of the curve might alter when different suspensions were used, changes in the buffer concentration always produced a curve which rose to a peak at the same buffer concentration in a system in which the cytochrome c concentration was adequate and constant.



22



23

Fig. 22.—Variations in the total oxygen uptake of the cytochrome system produced by electrolytes. Contents of the Warburg vessel were: 0.2 ml. of 0.4M phosphate buffer pH 7.3, 0.3 ml. of 1/30 rat brain suspension, 0.6 ml. of cytochrome c solution, 0.25 ml. of 0.1M 1-phenylsemicarbazide, and 0.25 ml. of 0.2M hydroquinone. Electrolytes added in varying amounts were: (1) 0.4M KCl, (2) 0.4M NaCl, (3) 0.4M Na_2SO_4 , (4) 0.4M sodium citrate. Total volume of 2.5 ml. made up with water.

Fig. 23.—The data in Figure 22 plotted to show the relationship between total oxygen uptake and the valencies of electrolytes added. For this purpose, 0.4M KCl or NaCl was taken as 0.4N, 0.4M Na_2SO_4 as 0.8N, and 0.4M sodium citrate as 1.2N. (1) KCl, (2) NaCl, (3) Na_2SO_4 , (4) sodium citrate.

If tissue suspension in water and a solution of cytochrome in water were mixed, a flocculent precipitate was observed to form. This coagulation could be prevented by adding a solution of NaCl, Na_2SO_4 , or phosphate buffer. It is not unlikely that this phenomenon is related to the influence of some electrolytes upon the O_2 uptake by the cytochrome system which has been described above.

(e) *The Effect of pH upon the System*

It was found that the O_2 uptake of the system reached a maximum within the pH range 7.1-7.3 (Figs. 24A and 24B). As the pH increased the total O_2 uptake increased. The autooxidation of the substrate, however, also became greater, as indicated by the magnitude of the intercept which the oxidase assay curve made on the "y" axis. When the values for the autooxidation were subtracted from the total oxygen uptakes (Fig. 24A), the net O_2 uptakes for the cytochrome system remained constant over the range of pH from 7.1 to 7.3. Below pH 7.1 the system was inhibited to an increasing degree as the conditions became more acid. At reactions higher than pH 7.5 the autooxidation of the substrate accounted for too large a proportion of the total O_2 uptake to allow the oxidase activity to be measured accurately (Fig. 24B).

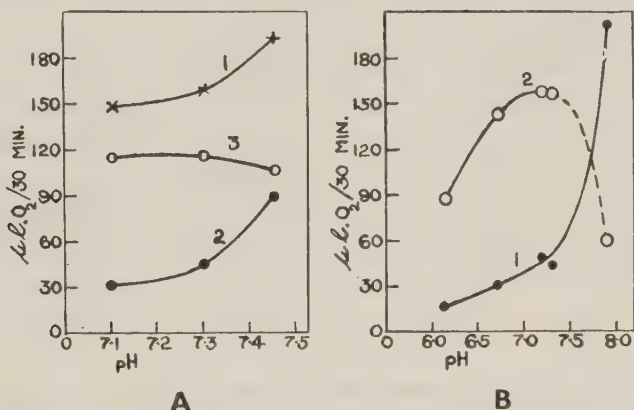


Fig. 24.—Relationship between pH and the efficiency of the cytochrome system. Different suspensions of 1/30 rat brain in A and B; oxygen uptake values for 30 minutes obtained from assay curves similar to those in Figures 1 and 2. A(1), total oxygen uptake for 0.15 ml. suspension; A(2), autooxidation or "y" intercept; A(3), net oxygen uptake due to cytochrome system (=A(1) minus A(2)); B(1), autooxidation or "y" intercept; B(2), net oxygen uptake or activity of cytochrome oxidase in 0.15 ml. suspension.

IV. RESUME

In the preceding sections a study was made of the factors which were found to influence the uptake of oxygen by the cytochrome system *in vitro*. It was shown that in order to estimate cytochrome oxidase activity the following precautions are necessary:

- (1) Tissues must be kept at 0°-4°C. if some time is to elapse before they can be examined for cytochrome oxidase content. They should be minced and handground in the homogeniser with distilled water, and the homogeniser should not be used for a longer period than 1 minute at 1,200-2,000 r.p.m. The suspension should be made up to volume with water, to effect plasmolysis, and after standing for approximately 20 minutes, the larger particles should be removed by straining through gossamer veil.

- (2) Cytochrome c should be freed from electrolytes by dialysis. The amount of cytochrome c necessary to ensure that the maximal O_2 uptake is achieved by the oxidase is estimated by cytochrome assay. The cytochrome solution should be boiled and filtered to ensure removal of impurities.
- (3) An assay of the phosphate buffer to be used should be carried out for each new preparation of cytochrome c, in order that the buffer concentration which will produce maximal O_2 uptake can be ascertained.
- (4) The final concentration of hydroquinone, which is used as a substrate, may be varied over a reasonable range without any effect upon the measurement of cytochrome oxidase, other than a change in the rate of autoxidation. It should obviously be kept constant in any one experiment.
- (5) The 1-phenylsemicarbazide should be dissolved in water at $100^\circ C.$ and maintained in solution at $37^\circ C.$ since it is only sparingly soluble in cold water. For this reason it is added when everything except tissue suspension is already in the Warburg vessels.
- (6) The shape of the vessels and the rate of shaking should clearly be such as to ensure that the rate of solution of oxygen in the liquid phase is not a limiting factor.

V. RECOMMENDED PROCEDURE

The procedure which is now followed in this laboratory for the estimation of the cytochrome oxidase content of a tissue is illustrated by a typical experiment the general arrangement of which is shown in Table 2. A preliminary cytochrome c assay and buffer curve had established the fact that the necessary quantities of each were 1.0 ml. and 0.5 ml. respectively. The tissue suspension was rat brain, 1 g. in 30 ml.

TABLE 2

Reagents	Vessel No.						
	1	2	3	4	5	6	7*
	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Water	0.23	0.20	0.15	0.10	0.05	nil	2.0
0.4M Sodium phosphate buffer, pH 7.1	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cytochrome c solution ($c. 0.5 \times 10^{-3}M$)	1.0	1.0	1.0	1.0	1.0	1.0	—
0.2M Hydroquinone in the side-arm	0.25	0.25	0.25	0.25	0.25	0.25	—
0.1M 1-Phenylsemicarbazide in the reaction chamber	0.5	0.5	0.5	0.5	0.5	0.5	—
Tissue suspension	0.02	0.05	0.10	0.15	0.20	—	—
Boiled tissue suspension = nil oxidase	—	—	—	—	—	0.25	—
Total liquid volume	2.5	2.5	2.5	2.5	2.5	2.5	2.5

*Thermobarometer

These quantities may be altered to suit the conditions, provided always that the cytochrome c and buffer concentration are optimal, the amount of tissue suspension is not excessive, and the concentrations of hydroquinone and 1-phenyl-semicarbazide are neither limiting nor so great as to be toxic.

The vessels are equilibrated 5 to 10 min. before tipping. The technique to be followed with reference to the readings has already been described above. Uptake-time curves are drawn (Fig. 1) and from these the O_2 uptakes at the end of 30 min. are extrapolated. These are plotted against ml. of suspension (Fig. 2). The value of the intercept on the "y" axis by the straight line so formed is taken as the autoxidation and subtracted from the O_2 uptake value read from the curve for 0.15 ml. of suspension to give the net O_2 uptake for 30 min. ($171 - 35 = 136 \mu l.$ in Fig 2). The dry matter in the suspension is determined by drying aliquots of 5 ml. each in an air oven at $100^\circ C.$ overnight. From the value for the net O_2 uptake for 30 min. and the value for the dry matter in 0.15 ml. of suspension, the QO_2 (O_2 uptake/hr./mg. dry matter of the tissue) is calculated.

If desired, a succinoxidase assay by the method of Schneider and Potter (1943) will give an estimate of the proportion of unbroken cells in the suspension. It is necessary to know this in order to apply a correction to the QO_2 , since only the cytochrome oxidase which has been liberated from the cells can be estimated by this method (Schneider and Potter 1943).

VI. ACKNOWLEDGMENTS

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The interest and help of colleagues at the Nutrition Laboratory was greatly appreciated. The authors are particularly indebted to Mr. H. R. Marston, Chief of the Division of Biochemistry and General Nutrition, C.S.I.R., for suggesting the work, for his constructive advice throughout, and for his criticism and help in the preparation of the manuscript.

VII. REFERENCES

- ALBAUM, H. G., TEPPERMAN, J., and BODANSKY, O. (1946a).—*J. Biol. Chem.* 163: 641.
 ——— (1946b).—*Ibid.* 164: 45.
 ALTSCHUL, A. M., ABRAMS, R., and HOGNESS, T. R. (1939). *Ibid.* 130: 427.
 DICKENS, F. (1946).—*Biochem. J.* 40: 145.
 KEILIN, D., and HARTREE, E. F. (1937).—*Proc. Roy. Soc. B* 122: 298.
 ——— (1938).—*Ibid.* 125: 171.
 MARSTON, H. R., QUINLAN-WATSON, T. A. F., and DEWEY, D. W. (1943).—*J. Coun. Sci. Industr. Res. Aust.* 16: 113.
 POTTER, V. R., and ELVEHJEM, C. A. (1936).—*J. Biol. Chem.* 114: 495.
 RIGGS, B. C. (1945).—*Ibid.* 161: 381.

- ROSENTHAL, O., and DRABKIN, D. L. (1943).—*J. Biol. Chem.* 149: 437.
- SCHNEIDER, W. C., and POTTER, V. R. (1943).—*Ibid.* 149: 217.
- SCHULTZE, M. O. (1939).—*Ibid.* 129: 729.
- STADIE, W. C., and HAUGAARD, N. (1945).—*Ibid.* 161: 153.
- STOTZ, E. (1939).—*Ibid.* 131: 555.
- , ALTSCHUL, A. M., and HOGNESS, T. R. (1938).—*Ibid.* 124: 745.

STUDIES ON THE NITROGEN METABOLISM OF PLANTS*

VIII. UTILIZATION OF α -OXIMINOCARBOXYLIC ACIDS BY OAT PLANTS

By J. G. WOOD† and MARY R. HONE†

[Manuscript received April 16, 1948]

Summary

Oat plants were grown in culture solutions containing hydroxylamine, oximinopropionic acid, *trans*-oximinosuccinic acid and *cis*- and *trans*-oximino-glutaric acids in different concentrations at pH 7. Morphological changes and nitrogen contents, including protein-N contents, of these plants are described. It is shown that all these oximes can be utilized as a source of nitrogen for protein synthesis; optimum concentrations for protein synthesis in oats is about 1×10^{-5} M for hydroxylamine, 1×10^{-4} M for oximinopropionate and 1×10^{-3} M for the oximino-dicarboxylic acids.

The amount of protein synthesis was small in plants grown in solutions of hydroxylamine and oximinopropionic acid which also caused marked depression in dry weight production, especially in roots. In solutions of oximino-dicarboxylic acids plants were normal and the amount of protein synthesis was relatively large, though less than that in plants grown in nitrate solutions of the same molarity.

The possibility that oximinosuccinate and oximinoglutarate are intermediaries in the formation of proteins from nitrate is briefly discussed.

I. INTRODUCTION

Vickery *et al.* (1940) have shown that when ammonium salts containing N¹⁵ are supplied to plants the isotope quickly appears in amino-acids, amides, and proteins within the plant. Data have accumulated which show that, so far as ammonium salts are concerned, mechanisms are present in plants whereby amino-acids and amides may be produced from ammonia and keto-acids—especially oxalacetic and α -ketoglutaric acids.

It has been generally held that nitrates are reduced in the leaf ultimately to ammonia. Recently Burström (1945) has claimed that in wheat leaves nitrate reduction is dependent on light intensity and suggested from his evidence that reduction of nitrate to ammonia did not occur, but that a direct carbon-nitrogen assimilate was formed which was probably an oxime; in this respect leaves differ from roots where nitrate reduction is not dependent on light. This view gains some support from Endres' (1935) isolation of a carboxime from *Azotobacter* and from Virtanen's (1939) claim that he had isolated β -oximinosuccinic

* Earlier papers in this series I-IV *Ann. Bot.* 2(1938); *Ibid.* 4(1941); V, VI, *Aust. J. Exp. Biol. Med. Sci.* 20(1942): pp. 249-56, pp. 257-62.

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acid from nodules of *Rhizobium*. Whether an oxime is an intermediary in the metabolism of these organisms has been the subject of controversy (Burris and Wilson 1945; Virtanen 1947) and Virtanen (1947) has recently suggested that in *Rhizobium* much of the nitrate supplied might be reduced to ammonia and that the oxime is formed in relatively small amounts. Wood *et al.* (1948) investigated the toxicity to *Azotobacter* of a number of oximino-derivatives and showed, *inter alia*, that α -oximinosuccinic and α -oximinoglutaric acids are relatively non-toxic and furthermore that *Azotobacter* could utilize these compounds as a source of nitrogen when deprived of all other forms of that element. In this paper we have investigated the utilization by oat plants of hydroxylamine, oximinopropionic acid, oximinosuccinic acid, and oximinoglutaric acids.

II. METHODS

(a) Cultural and Analytical

A pure line of oats (*Avena sativa* var. Mulga) was used. Seeds were germinated on waxed mosquito netting stretched over porcelain dishes filled with distilled water. After 10-14 days the seedlings were transferred to a basal culture solution in glass museum jars of 2.5 litres capacity which were coated externally with black paint and internally with paraffin wax. Six even seedlings were placed in each jar; they were inserted through holes in a wooden cover-block which, in each experiment, was freshly coated with paraffin wax; the seedlings were held in place by means of paraffined cotton wool. Glass distilled water was used throughout and the solutions were aerated three times daily.

The basal nutrient solution had the following composition in g. per litre of distilled water, all salts being of A.R. grade: KCl, 0.5 g.; KH_2PO_4 , 0.25 g.; K_2HPO_4 , 0.25 g.; NaCl, 0.1 g.; $\text{CaSO}_4 \cdot \text{H}_2\text{O}$, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; Fe_2Cl_6 , 0.02 g.; H_3BO_3 , 0.5 mg.; 0.5 mg. Mn as $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$; 0.2 mg. Zn as ZnSO_4 ; 0.1 mg. Cu as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; 0.25 mg. Mo as $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$. The pH value of the solution was usually pH 7.0.

Growth of seedlings was continued in the basal solution until N-deficient symptoms appeared, i.e. yellowing of tips of first-formed leaves. At this stage appropriate treatments of the different nitrogen compounds were added; the oximino-acids were dissolved in water neutralized with NaOH and made up to convenient concentration before addition to the basal solution.

Oximinopropionic acid, *trans*-oximinosuccinic acid, and *cis*- and *trans*-oximinoglutaric acids were synthesized and purified by the methods described by Wood *et al.* (1948).

In order to avoid possible contamination and also decomposition, the nutrient solutions (including the basal) were changed frequently—usually every two or three days.

The plants were grown in a glasshouse until harvest when a typical plant from each treatment was carefully drawn to scale; the remainder were harvested, roots and shoots separated and dried quickly under forced draught at 90°C.

Protein-N and soluble-N were determined by micro-Kjeldahl after extraction of dried material and precipitation of protein with trichloroacetic acid at pH 4.5 according to the method described by Hanson, Barrien, and Wood (1941).

(b) *The Problem of Absorption of Oximino-Acids by Plants*

It is difficult to show that the oximino-acids have been absorbed as such by plants. Endres (1935) and Lemoigne, Monguillon, and Desveaux (1936) have described methods for estimation of free and "combined" hydroxylamine (oxime), based on oxidation to nitrite of freed hydroxylamine, by heating the latter with iodine in glacial acetic acid and subsequent diazotization and coupling with sulphanilic acid and α -naphthylamine (Griess reagent). Steward and Street (1947) have referred to some difficulties in applying the method to plant tissue, but give a table indicating the presence of both free and "combined" hydroxylamine in leaves of legumes. We have not estimated oxime-nitrogen in the plants because we do not believe that the above methods are capable of measuring oxime-nitrogen in the dicarboxylic acids we have employed. It is probable that the methods are applicable with α (*cis*)-oximinoglutaric acid which is relatively stable in slightly acid as well as in alkaline solution and can be recovered from boiling aqueous solution. However, like Cramer (1891), we have found that β (*trans*)-oximinosuccinic acid is quite stable in neutral or alkaline solutions but is extremely labile in acid solution; even at room temperatures an aqueous solution of the pure acid decomposes rapidly, losing carbon dioxide and water and forming cyanoacetic acid which can be recovered as red crystals; in alkaline solution cyanoacetic acid readily forms malonic acid and ammonia. In neutral or alkaline solutions β (*trans*)-oximinoglutaric acid is stable, but is labile in acid solution; even on gentle warming a pure aqueous solution of the acid loses carbon dioxide and is converted into the half-amide of succinic acid. It is obvious, therefore, that the methods described above will not free hydroxylamine from these acids which can then be oxidized to nitrite.

The culture solutions were approximately neutral and were changed frequently in order to prevent occurrence of the changes outlined above and also to minimize possible bacterial contamination. Investigations were carried out from time to time on the culture solutions. Large volumes have been distilled under reduced pressure and have failed to yield any ammonia. The possibility that the oxime group of the oximino-acids might be oxidized to nitrite or nitrate (e.g. by ferric chloride present) has also been investigated. Using Rider and Mellon's (1946) quantitative modification of the Griess reagent, it has not been possible to detect the presence of nitrite or of nitrate in our culture solutions which were up to three days old. With *cis*-oximinoglutaric acid, however, small amounts of nitrite were detected in solutions of pH 6.8, and containing $1.0 \times 10^{-3}M$ or more of the oximino-acid, which were four days old; the concentration of nitrite on the

fifth day was $2 \times 10^{-8}\text{M}$, on the seventh day the concentration was $2 \times 10^{-7}\text{M}$, and on the eleventh day $1 \times 10^{-6}\text{M}$. These amounts are too small to provide detectable changes in nitrogen contents of the plants. For example, in Experiment 6, described below, the solutions, in contrast to all other experiments, were changed only weekly, since over the whole experimental time, breakdown of the oxime only provided 0.05 mg. N as nitrite in the culture solutions and available to the plants. It is considered reasonably certain, therefore, that hydrolytic or oxidative changes in the solutions under the conditions described were negligible and that the changes in nitrogen fractions within the plant were brought about following absorption of the oximino-acids.

III. RESULTS

(a) *Growth of Oats on Hydroxylamine and Oximinopropionate*

Experiment 1.—A preliminary experiment was carried out in order to determine the morphological effects and toxicity of different concentrations of oximino-derivatives. Oat seedlings were grown as described until they were N-deficient, when hydroxylamine, oximinopropionic acid, and *trans*-oximinosuccinic acid were added to the basal solution each in quantities to give final molar concentrations of 1×10^{-6} , 5×10^{-6} , 1×10^{-5} , 5×10^{-5} , and $1 \times 10^{-4}\text{M}$. Control pots containing no added oxime and also others containing $1 \times 10^{-4}\text{M}$ potassium nitrate were prepared at the same time. The pH of the culture solution in each case was adjusted to pH 7 with NaOH solution and each culture solution was completely renewed each day for 30 days; at the end of 30 days the results outlined below were observed.

Over the range of concentrations investigated *trans*-oximinosuccinic acid produced no toxic symptoms; development of roots and shoots was normal although plants were obviously N-deficient at the lower concentrations.

With hydroxylamine and oximinopropionate toxic symptoms in plants were similar. At all concentrations stunting of the root system occurred, even when compared with control plants grown on solutions with no added nitrogen; at concentrations higher than $1 \times 10^{-5}\text{M}$ with hydroxylamine and $5 \times 10^{-5}\text{M}$ with oximinopropionate the roots were brown in colour and no lateral roots developed; at the concentrations mentioned the roots were still brownish in colour with few laterals; at lower concentrations roots were not discoloured and development of laterals occurred.

The effect on the shoot system was less marked. At the higher concentrations leaf development and tillering were suppressed; incipient tillers developed on plants in $1 \times 10^{-5}\text{M}$ hydroxylamine and in $5 \times 10^{-5}\text{M}$ oximinopropionate and at slightly lower concentrations tillering occurred even though the root system remained stunted.

The concentrations of hydroxylamine and oximinopropionate at which tillering and lateral root development were suppressed were approximately the same as those found toxic to *Azotobacter* by Wood *et al.* (1948).

Experiment 2.—Duplicate sets of oat seedlings were grown as described previously in basal solutions containing oximinopropionate, potassium nitrate, and no added nitrogen, at the concentrations and under conditions set out in Table 1. The solutions were adjusted to pH 7 and completely renewed every two days. One set was harvested after plants had grown for 20 days in the oximinopropionate solutions and the other set after 34 days. Results of analyses for dry weight, protein-N and soluble-N are given in Table 1 and typical plants from each treatment after 34 days are illustrated in Figure 1.

TABLE 1

DRY WEIGHTS (G./5 PLANTS), PROTEIN-N, SOLUBLE-N, AND TOTAL-N (MG./5 PLANTS) OF OAT PLANTS GROWN IN NUTRIENT SOLUTIONS AT pH 7 WITH α -OXIMINOPROPIONIC ACID AS SOLE SOURCE OF NITROGEN

Molarity	Shoots				Roots		Total Plant	
	D.W. (g.)	Prot-N (mg.)	Sol-N (mg.)	Tot-N (mg.)	D.W. (g.)	Tot-N (mg.)	D.W. (g.)	Tot-N (mg.)
<i>Series A*</i>								
Nil (Control)	0.145	1.80	0.84	2.64	0.132	0.30	0.277	2.94
7.0×10^{-5} M Oxime	0.190	3.22	1.28	4.50	0.091	0.31	0.281	4.81
1.4×10^{-4} M Oxime	0.184	2.37	1.62	3.99	0.090	0.26	0.279	4.25
3.5×10^{-4} M Oxime	0.166	1.93	0.77	2.70	0.074	0.23	0.240	2.93
7.0×10^{-4} M Oxime	0.173	2.13	0.82	2.95	0.069	0.10	0.242	3.03
3.5×10^{-4} M KNO_3	0.261	10.14	3.00	13.10	0.139	0.51	0.400	13.61
<i>Series B†</i>								
Nil (Control)	0.233	2.42	0.27	2.69	0.178	0.34	0.401	3.03
7.0×10^{-5} M Oxime	0.263	3.47	1.53	5.00	0.094	0.29	0.357	5.29
1.4×10^{-4} M Oxime	0.284	4.33	1.17	5.55	0.101	0.31	0.385	5.86
3.5×10^{-4} M Oxime	0.226	2.28	1.17	3.45	0.075	0.23	0.301	3.68
7.0×10^{-4} M Oxime	0.214	2.49	1.13	3.62	0.078	0.16	0.292	3.78
3.5×10^{-4} M KNO_3	0.892	35.10	9.68	44.80	0.278	1.90	1.170	46.70

* Plants 20 days in oxime solutions. All solutions changed every two days.

† Plants 34 days in oxime solutions.

Experiment commenced 3.v.46.

In appearance the plants were similar to those described in Experiment 1. Root development was poor even when compared with the control receiving no extra N; incipient tillering and lateral root development occurred at 1.4×10^{-4} M and both were obvious at 7×10^{-5} M. The analyses show that, compared with the *minus-N* control, the shoots increased in dry weight in all treatments, being greatest at 1×10^{-4} M and 7×10^{-5} M. However, root weight increment was suppressed; root dry weights of plants exposed to oximinopropionate were less than that of the *minus-N* control; also no change in dry weight of the roots of these plants occurred between the 20th and 34th days.

Compared with the *minus-N* control the protein-N of the shoots of treated plants increased in amount in 7×10^{-5} M and 1.4×10^{-4} M solutions of oximinopropionate, being greatest in the former concentration after 20 days and in the

latter after 34 days; at other toxic concentrations no protein synthesis occurred. In roots the total-N was less in treated plants than in the control and no change in N-content occurred between the 20th and 34th days.

The evidence suggests, therefore, that at concentrations of about $1 \times 10^{-4}M$ shoots but not roots of oat plants can utilize oximinopropionic acid to synthesize proteins, though the extent of such synthesis is small compared with plants grown on nitrate solutions.



Fig. 1.—Appearance of typical plants grown in basal solution containing the following nitrogenous treatments: (a) no added nitrogen; (b) $7.0 \times 10^{-5}M$ α -oximinopropionate; (c) $1.4 \times 10^{-4}M$ α -oximinopropionate; (d) $3.5 \times 10^{-4}M$ α -oximinopropionate; (e) $7.0 \times 10^{-4}M$ α -oximinopropionate; (f) $3.5 \times 10^{-4}M$ potassium nitrate. (One-sixth natural size.)

Experiment 3.—The technique employed was as described previously. Three different treatments with hydroxylamine were supplied and also oximinopropionic acid, alanine, ammonium sulphate, and potassium nitrate in solutions of equal molarity. Details of concentration and treatment are shown in Table 2. Typical plants after 38 days in the culture solutions are illustrated in Figure 2 and results of analyses are shown in Table 2.

TABLE 2

DRY WEIGHTS (G./6 PLANTS), PROTEIN-N, SOLUBLE-N, AND TOTAL-N (MG./6 PLANTS) OF OAT PLANTS* GROWN IN NUTRIENT SOLUTIONS AT pH 6.8 WITH HYDROXYLAMINE AND OTHER FORMS OF NITROGEN

Molarity	Shoots				Roots		Total Plant	
	D.W. (g.)	Prot-N (mg.)	Sol.-N (mg.)	Tot.-N (mg.)	D.W. (g.)	Tot.-N (mg.)	D.W. (g.)	Tot.-N (mg.)
Nil (Control)	0.277	3.27	1.22	4.49	0.231	1.96	0.508	6.45
7.0×10^{-5} M Hydroxyl- amine†	0.241	3.24	1.88	4.12	0.133	2.03	0.374	6.15
3.5×10^{-5} M Hydroxyl- amine†	0.242	6.75	2.71	9.46	0.117	2.34	0.359	11.80
3.5×10^{-4} M Hydroxyl- amine	0.239	1.27	1.18	2.45	0.111	1.15	0.350	3.60
3.5×10^{-4} M Oximino- propionic	0.231	2.31	1.06	3.37	0.102	1.30	0.333	3.67
3.5×10^{-4} M Alanine	0.768	26.50	11.40	37.90	0.293	13.20	1.061	51.10
3.5×10^{-4} M $(\text{NH}_4)_2\text{SO}_4$	0.564	25.50	13.20	38.70	0.238	13.10	0.802	51.80
3.5×10^{-4} M KNO_3	1.274	18.90	7.20	26.10	0.498	14.90	1.772	41.00

* Plants 38 days in nitrogenous solutions.

† Solutions changed daily; remainder every three days.

Experiment commenced 4.vi.46.

From these it appears that hydroxylamine in 3.5×10^{-5} M concentration can be utilized by shoots of oat plants to form protein-N, the amount of synthesis being small, possibly owing to the dilution. At this concentration plants produce tillers but there is suppression of dry weight increase and no nitrogen synthesis is apparent in roots. The behaviour of hydroxylamine, therefore, is apparently similar to that of oximinopropionic acid but is toxic at lower concentrations.

TABLE 3

DRY WEIGHTS (G./6 PLANTS), PROTEIN-N, SOLUBLE-N, AND TOTAL-N (MG./6 PLANTS) OF OAT PLANTS* GROWN IN NUTRIENT SOLUTIONS AT pH 7 WITH TRANS-OXIMINOSUCCINIC ACID AS SOLE SOURCE OF NITROGEN

Molarity	Shoots				Roots		Total Plant	
	D.W. (g.)	Prot-N (mg.)	Sol.-N (mg.)	Tot.-N (mg.)	D.W. (g.)	Tot.-N (mg.)	D.W. (g.)	Tot.-N (mg.)
Nil (Control)	0.193	2.69	0.99	3.78	0.180	1.82	0.373	5.60
3.5×10^{-5} M Acid†	0.223	4.25	1.62	5.87	0.200	2.88	0.423	8.75
7.0×10^{-5} M Acid	0.235	4.63	2.00	6.63	0.207	3.21	0.442	9.84
3.5×10^{-4} M Acid	0.240	4.83	2.99	7.82	0.206	3.54	0.446	11.36
7.0×10^{-4} M Acid	0.319	9.62	4.28	13.90	0.204	5.77	0.523	19.67
3.5×10^{-4} M KNO_3	0.490	13.62	4.14	17.76	0.490	5.50	0.980	23.26

* Plants 36 days in nitrogenous solutions.

† Solutions changed daily; remainder every three days.

Experiment commenced 5.vii.46.

Other treatments in this series (Fig. 2 and Table 2) emphasize the differences when plants were grown in solutions of equal molarity of oximinopropionic acid, alanine (the amino-acid corresponding to oximinopropionic acid), ammonium sulphate, and potassium nitrate.

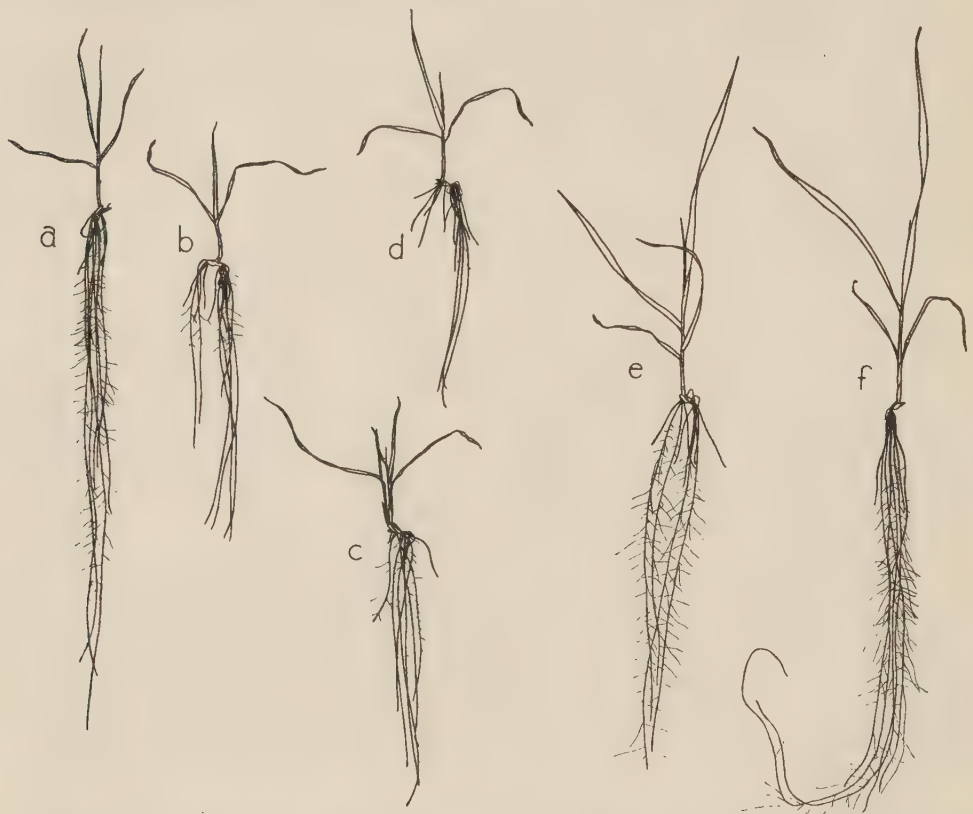


Fig. 2.—Appearance of typical plants grown in basal solution containing the following nitrogenous treatments: (a) no added nitrogen; (b) $7.0 \times 10^{-5}M$ hydroxylamine; (c) $3.5 \times 10^{-5}M$ hydroxylamine; (d) $3.5 \times 10^{-4}M$ α -oximinopropionate; (e) $3.5 \times 10^{-4}M$ alanine; (f) $3.5 \times 10^{-4}M$ potassium nitrate. (One-sixth natural size.)

(b) *Growth of Oats on β (trans)-Oximosuccinate*

Experiment 4.—Concentrations of oximosuccinate and potassium nitrate used and experimental conditions employed are shown in Table 3. Typical plants from each treatment after 36 days are illustrated in Figure 3 and results of analyses of plant material are shown in Table 3.

In appearance the plants were strikingly different from those grown in hydroxylamine and oximinopropionate, and described previously; they confirm the results obtained in Experiment 1. The root systems in all cases were normal although at all oxime concentrations the dry weights of the roots were probably not significantly greater than that of the control; however, increase in dry weight of the shoots occurred with increased oxime concentration.

Protein synthesis occurred in both shoots and roots, and in all cases the protein content of the plants increased as the concentration of oximinosuccinate increased. Plants grown in $3.5 \times 10^{-4}\text{M}$ oximinosuccinate contained less (about half) nitrogen than those grown in potassium nitrate at the same concentration, but plants grown on $7 \times 10^{-4}\text{M}$ oximinosuccinate were comparable, so far as N-content was concerned, with those grown in nitrate at the lower concentration.

It appears, therefore, that oat plants utilize oximinosuccinate readily as a source of nitrogen.



Fig. 3.—Appearance of typical plants grown in basal solution containing the following nitrogenous treatments: (a) no added nitrogen; (b) $3.5 \times 10^{-5}\text{M}$ *trans*-oximinosuccinate; (c) $7.0 \times 10^{-5}\text{M}$ oximinosuccinate; (d) $3.5 \times 10^{-4}\text{M}$ oximinosuccinate; (e) $7.0 \times 10^{-4}\text{M}$ oximinosuccinate; (f) $3.5 \times 10^{-4}\text{M}$ potassium nitrate. (One-sixth natural size.)

(c) Growth of Oats on *cis*- and *trans*-Oximinoglutarate

Experiment 5.—Three treatments, including *trans*-oximinoglutaric acid, were applied as shown in Table 4 where conditions are also set out. Typical plants from each treatment are illustrated in Figure 4 and results of analyses are given in Table 4.

The results were similar to those described above for plants grown in oximosuccinate. Roots and shoots were normal in appearance and it is apparent from Table 4 that *trans*-oximinoglutarate is utilized for protein synthesis by oats.

Experiment 6.—Oat plants were grown in solutions containing *cis*-oximinoglutarate and potassium nitrate at the concentrations and under conditions shown in Table 5.



Fig. 4.—Appearance of typical plants grown in basal solution containing the following nitrogenous treatments: (a) no added nitrogen; (b) $3.5 \times 10^{-4}M$ *trans*-oximinoglutarate; (c) $3.5 \times 10^{-4}M$ potassium nitrate. (One-sixth natural size.)

The appearance of the plants after 54 days is illustrated in Figure 5 and results of analyses are set out in Table 5. The treatments included concentrations higher than those previously employed.

TABLE 4

DRY WEIGHTS (G./6 PLANTS), PROTEIN-N, SOLUBLE-N, AND TOTAL-N (MG./6 PLANTS) OF OAT PLANTS* GROWN IN NUTRIENT SOLUTIONS AT pH 7 WITH TRANS-OXIMINOGLUTARIC ACID AS SOLE SOURCE OF NITROGEN

Molarity	Shoots				Roots		Total Plant	
	D.W. (g.)	Prot-N (mg.)	Sol-N (mg.)	Tot-N (mg.)	D.W. (g.)	Tot-N (mg.)	D.W. (g.)	Tot-N (mg.)
Nil (Control)†	0.213	3.70	1.04	4.74	0.195	1.49	0.408	6.23
3.5×10^{-4} M Acid	0.287	6.77	2.52	9.29	0.207	4.06	0.494	13.35
3.5×10^{-4} M KNO_3	0.419	13.60	4.40	15.00	0.196	4.52	0.615	19.52

* Plants 34 days in nitrogenous solutions.

† All solutions changed every three days.

Experiment commenced 20.vii.46.

TABLE 5

DRY WEIGHTS (G./6 PLANTS), PROTEIN-N, SOLUBLE-N, AND TOTAL-N (MG./6 PLANTS) OF OAT PLANTS* GROWN IN NUTRIENT SOLUTIONS AT pH 6.8 WITH CIS-OXIMINOGLUTARIC ACID AS SOLE SOURCE OF NITROGEN

Molarity	Shoots				Roots		Total Plant	
	D.W. (g.)	Prot-N (mg.)	Sol-N (mg.)	Tot-N (mg.)	D.W. (g.)	Tot-N (mg.)	D.W. (g.)	Tot-N (mg.)
Nil (Control)	0.094	0.74	0.76	1.50	0.132	1.35	0.226	2.85
5×10^{-4} M Acid	0.132	2.58	2.76	5.34	0.096	2.46	0.228	7.80
1×10^{-3} M Acid	0.198	4.32	3.54	7.86	0.106	3.06	0.304	10.92
5×10^{-3} M Acid	0.166	2.70	1.86	4.56	0.086	2.76	0.252	7.32
5×10^{-4} M KNO_3	0.214	4.74	2.22	6.96	0.094	2.00	0.308	8.96
1×10^{-3} M KNO_3	0.262	4.80	2.76	7.56	0.104	2.52	0.366	10.08
5×10^{-3} M KNO_3	0.560	14.52	6.72	21.24	0.224	4.86	0.794	26.10

* Plants 54 days in nitrogenous solutions. All solutions changed every ten days.

Experiment commenced 10.vii.47.

That *cis*-oximinoglutarate is more toxic in 5×10^{-3} M solution than in 1×10^{-3} M is reflected in the morphology of the plants and in their dry weight and protein contents, although N-synthesis occurred at the higher concentration. At the two lower concentrations of *cis*-oximinoglutarate root dry weight was approximately the same as that of plants grown in nitrate of the same molarity, although in the oxime solutions the root system was more compact. In shoots, dry weight was less in plants grown in oximes than those in nitrates, but protein synthesis occurred readily in plants grown in the oximinoglutarate solutions.



Fig. 5.—Appearance of typical plants grown in basal solution containing the following nitrogenous treatments: (a) no added nitrogen; (b) 5×10^{-4} M potassium nitrate; (c) 1.0×10^{-3} M potassium nitrate; (d) 5.0×10^{-3} M potassium nitrate; (e) 5×10^{-4} M *cis*-oximinoglutarate; (f) 1.0×10^{-3} M *cis*-oximinoglutarate; (g) 5.0×10^{-3} M *cis*-oximinoglutarate. (One-sixth natural size.)

IV. DISCUSSION

The validity of the results described here depends upon showing that the oximino-acids are absorbed unchanged by plants. For reasons discussed earlier this is difficult to establish by analyses of plants but it has been shown that, under the conditions used, the oximes did not undergo reduction, oxidation, or hydrolysis in the culture solutions prior to adsorption by the plants.

The experiments described indicate that in non-toxic concentrations hydroxylamine, oximinopropionate, *trans*-oximinosuccinate, and both *cis*- and *trans*-oximinoglutarate can all be utilized by oat plants as sources of nitrogen to form proteins. With *trans*-oximinosuccinate and *trans*-oximinoglutarate the highest concentrations employed were respectively 7×10^{-4} M and 3.5×10^{-4} M, at both of which concentrations there was no evidence of toxic symptoms in plants grown in them. The optimum concentrations for protein synthesis appear to be about 1×10^{-5} M for hydroxylamine, about 1×10^{-4} M for oximinopropionic acid, and about 1×10^{-3} M for the three oximino-dicarboxylic acids which have been investigated.

Plants grown in both hydroxylamine and oximinopropionate solutions show marked depression of dry weight compared with control plants and this is probably connected with the known effect of hydroxylamine on photosynthesis; the depression in dry weight production is more marked in roots than in shoots.

With the oximino-dicarboxylic acids dry weight changes in plants, especially in shoots, are less obvious; the percentage protein content on a dry weight basis is approximately the same in plants grown in the oximino-dicarboxylic acids and nitrate solutions of equal molarity; this suggests interdependence of the processes of protein formation and dry weight production which is not apparent in plants grown in solutions of hydroxylamine and oximinopropionate.

The amount of protein formed in plants grown in solutions containing the oximino-dicarboxylic acids is relatively large and in some cases approaches that of plants grown on nitrate solutions of the same molarity.

The greater amount of growth and of protein synthesis in shoots compared with roots tend to support Burström's (1945) suggestion, discussed earlier, that oxime production occurs in leaves rather than in roots.

The interest in oximinosuccinate and oximinoglutarate and their ready utilization by oat plants for protein synthesis lies in their relationship with the corresponding keto-acids oxalacetic and α -ketoglutaric acids, and with the corresponding amino-acids aspartic and glutamic acids. Both these oximino-dicarboxylic acids are possible intermediates in the formation of amino-acids from keto-acids when nitrogen is supplied as nitrate. Figures published by Steward and Street (1947) for leaves of bean plants suggest connection between oxime and keto-acid contents, although for reasons described earlier such figures for oxime-nitrogen may have little meaning. At the present state of knowledge speculation seems idle and the results described in this paper do no more than show that the oximino-dicarboxylic acids readily, and oximinopropionic acid and hydroxylamine in non-toxic concentration, can be utilized by oat plants to synthesize proteins.

V. REFERENCES

- BURRIS, R. H., and WILSON, P. N. (1945).—*Ann. Rev. Biochem.* **14**: 685.
BURSTRÖM, H. (1945).—*Ann. Roy. Agric. Coll. Sweden* **13**: 1.
CRAMER, C. (1891).—*Ber. dtsch. chem. Ges.* **24**: 1198.
ENDRES, D. (1935).—*Liebigs Ann.* **518**: 109.
HANSON, E. A., BARRIEN, B.S., and WOOD, J. G. (1941).—*Aust. J. Exp. Biol. Med. Sci.* **19**: 231.
LEMOIGNE, M., MONGUILLON, P., and DESVEAUX, R. (1936).—*Bull. Soc. Chem. Biol.* **18**: 840.
RIDER, B. F., and MELLON, M. G. (1946).—*Industr. Engng. Chem.* **18**: 96.
STEWART, F. C., and STREET, H. E. (1947).—*Ann. Rev. Biochem.* **16**: 471.
VICKERY, H. B., PUCHER, G. W., SCHOENHEIMER, R., and RITTENBERG, D. (1940).—*J. Biol. Chem.* **135**: 531.
VIRTANEN, A. I. (1939).—*Trans. 3rd Comm. Int. Soc. Soil Sci.*, A, p. 4.
VIRTANEN, A. I. (1947).—*Biol. Rev.* **22**: 239.
WOOD, J. G., HONE, M. R., MATTNER, M. E., and SYMONS, G. P. (1948).—*Aust. J. Sci. Res.* **B 1**: 38.

A STUDY OF CERTAIN ASPECTS OF THE ECOLOGY OF THE INTER-TIDAL ZONE OF THE NEW SOUTH WALES COAST

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(Plates 1-9)

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Summary

This paper is the result of an ecological study pursued for several years and extending over the entire length of the New South Wales coast. It is not a systematic investigation of one limited locality, but an attempt to analyse basically a long stretch of the east Australian coast.

A basic and generally clear-cut zonation of plant and animal types has been recognized and described for the intertidal region of the rocky shores and the strip immediately above high-water mark. A series of indicator types is named and discussed with particular reference to the chief animal communities.

1. INTRODUCTION

The zoology of the intertidal zone of the coast of New South Wales (or, indeed, any part of the Australian coast) has received, up to date, very unbalanced attention. Parts of our coast—the ocean and estuarine shores near Sydney—have probably been as carefully “collected over” by systematists as any coast of the world. Other localities on the Australian coast have also been visited by expeditions with the sole aim of making collections, except in the Barrier Reef area where physiological studies of great interest were made. But, on the whole, non-taxonomic studies of the Australian species of marine animals have been rare whether the subject be ecology, behaviour, general life-histories, or even anatomy. Very little is known of the life-histories of the common shore species of echinoderms, molluscs, ascidians, etc., or of their general zoology.

The systematics of the Mollusca of the New South Wales coast has received considerable attention (Hedley, Iredale, Allan, and others) and, thanks to the late H. L. Clark of the United States, the distribution of the echinoderms for all Australia is relatively well known. It is, however, surprising, under the circumstances, that the animal communities of the intertidal zone have not aroused greater interest. The interrelationships of the more common types and the rela-

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tions between them and the environment offer much of great interest, especially at the present time when the results of similar studies in other parts of the world are becoming available.

The absence of such studies is noticeable, more especially since the late Charles Hedley pioneered the way in a stimulating address on "An Ecological Sketch of the Sydney Beaches" in 1915 and Johnston discussed certain ecological aspects of the littoral fauna and flora of Caloundra on the south Queensland coast in 1917. These papers although valuable, are somewhat limited in their ecological treatment. A more detailed study of one particular locality on our coast is that by Pope (1943) which deals with the ocean rock platform of Long Reef.

Hedley (1915) discusses a few of the common species of three different types of locality—the ocean sand beach, the muddy estuary, and the ocean reef—and refers to the fact that there are different zones in the intertidal area, and he frequently indicates how a species prefers a certain kind of niche on the shore. The present paper takes a different view-point. The authors set out with the intention of studying the relationships of the most common shore animals which act as indicators of certain zones between tide marks. By covering the whole of the New South Wales coast, and a stretch approximately 1,000 miles north and south, they planned to determine whether the considerable difference in latitude was reflected on the shores in the distribution of these basic types. It should be emphasized that the northern and southern political boundaries of New South Wales are not natural biological boundaries. The reason for the limits chosen in this paper is that they were the convenient end-points for the first part of a study working out from Sydney. However, there is a little more than this since the northern border of New South Wales is not far from a point where the intertidal fauna of eastern Australia suffers a definite change, and the southern border is not far from a long stretch of sandy coast. To have gone further afield in a southerly direction would practically have meant visiting the southern coast of Victoria which was not possible at the time.

Considerable differences were found in the nature of the fauna in such different types of localities as estuaries, sandy bays, mangrove covered inlets and so on, but the rocky ocean shore has presented a clear sequence of zones which is almost the same from north to south over more than the length of coast especially chosen. This important finding led to the restriction of attention in this paper to the rocky ocean shores.

However, before describing the distribution of the life on our rocky coasts, or any zonation which may be present, it is necessary to supply a few facts in regard to the physiography of the shores studied and to refer to certain physical factors which may be involved.

2. NOTES ON THE PHYSIOGRAPHY OF THE ROCKY COAST AT THE LOCALITIES SELECTED FOR STUDY

The coast of New South Wales is marked by a series of rocky headlands which vary in height and geological formation and often present very steep cliffs to the sea. In places these headlands are close, and small bays with pocket sand beaches are found between them. In other localities the coastline is low plain and there may be long and beautiful sand beaches extending for several miles between one rocky headland and the next. Such a beach is that extending

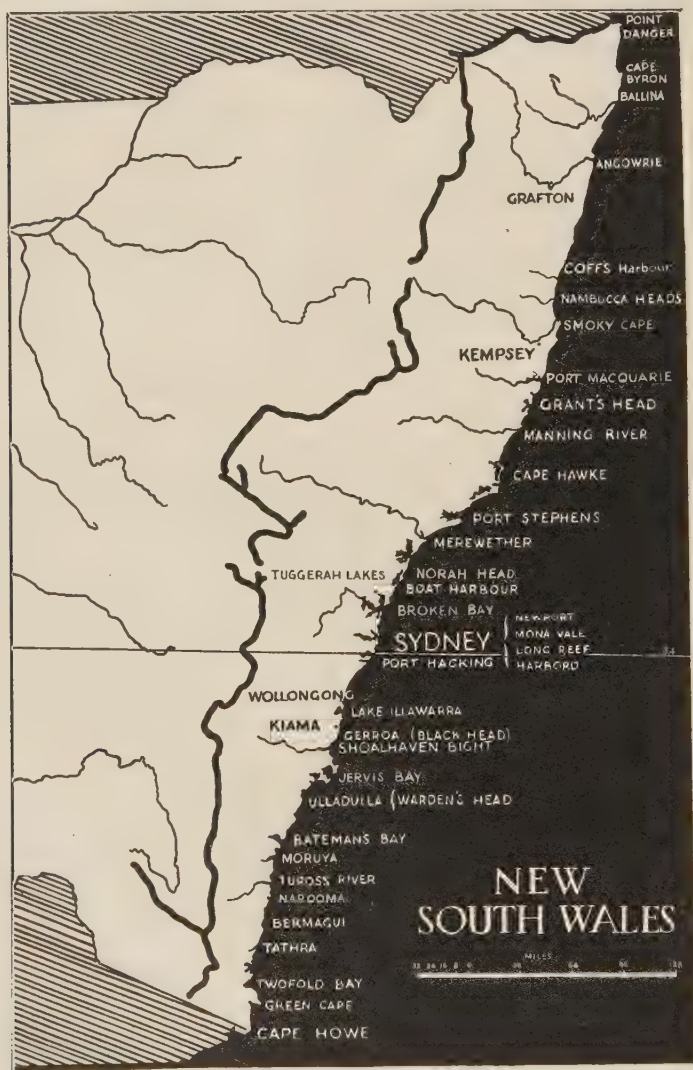


Fig. 1.—Map of the coast of New South Wales showing places mentioned in text.

for eleven miles immediately south of Smoky Cape, or the Seven Mile Beach to the north of the mouth of the Shoalhaven River.

At the foot of very many of the headland cliffs there are highly characteristic rock platforms, many of which are near sea-level, i.e. intertidal (see Plate 1). These rock platforms provide the best conditions for the present study.

They may be seen at Tweed Heads on the Queensland border, at Yamba and Angowrie in the north, and at various places as one passes south. Perhaps the best developed, and certainly the best known (almost famous) are in the vicinity of Sydney and these have been selected for the basic work, but there are other excellent platforms which are very good collecting grounds, such as those at Nambucca Heads and Norah Head (to the north), and at Black Head, Gerroa, and Warden's Head, Ulladulla (south of Sydney).

The rock platforms at Norah Head, Newport, Mona Vale, and Long Reef are broad, fairly flat expanses of rock which fringe the headlands and are left bare as the tide recedes. They are not level. Often they slope seaward and the ocean edge of the platform may be several feet lower than the level on the cliff-side. Sometimes one end of a rock platform is higher than the other. They may rise from the sea by a series of "steps" each several feet high as at Norah Head, Black Head, and also at Harbord and along the eastern edge of Long Reef. At Norah Head the platform margin is 40 ft. above sea-level at the extreme eastern point. At Long Reef again the eastern tip is the highest part and reaches 14-15 ft. but this high area is very small. The surfaces of the platforms are almost always uneven and broken up with hollows and cracks. Both the latter result in the formation of rock pools which are favoured haunts of animal life. These are especially interesting where blocks of rock of small size are resting, either on rock or on pockets of gravel and sand. (By small size, we mean a size reasonable enough to be turned over, but heavy enough, or wedged in some hollow, so as to remain undisturbed by the waves, except during storms or very heavy seas.)

The nature of the animal communities on these rock platforms depends firstly on their levels and secondly on the degree of exposure of the rock surface to the sea. This latter is affected by the orientation of the rocky headland. A point at or very near the margin of the rock platform will be differently affected by the sea from a point at the same tide level, but some distance in from the outer margin. Again, a rock platform margin facing north or south on the sides of a headland jutting out eastward to the sea will usually be less violently attacked by the surf than the extreme seaward point at the eastern extremity. There are other factors concerned in determining the distribution of intertidal life, but tide level and degree of exposure are obvious factors and they draw especial attention to the physiographic character of the rock platforms, and the nature of the rocky shore itself.

It is generally accepted that our coast is a relatively young coast and a coast of submergence. This implies that the coastal margin has been flooded in relatively recent times (geologically speaking) giving rise to estuaries like those of Port Jackson and the Hawkesbury River.

Where high ground was brought to the proximity of the sea the ocean has acted upon it, producing cliffs, cutting in and removing the debris resulting from atmospheric weathering and wave action, and as a result of this we find the steep cliffs of today with the rock platforms at their base. These rock platforms are very characteristic features of the coast of New South Wales.

Geomorphological text-books give typical diagrams of this cutting of rock platforms and these are sometimes pictured with the surfaces near high-tide level. Unfortunately, we meet some argument and a marked divergence in views as to the exact conditions under which our New South Wales rock platforms were, or are, being formed (see Cotton 1947; Gulliver 1898; Hedley 1924; Jardine 1925; Johnson 1919; Jutson 1939; Steers 1929; Wentworth 1938).

The basic idea is that at some particular level the sea has exerted its most effective powers and in combination with weathering has cut back the land, forming a platform and a sea-cliff. There is no doubt that the rock platforms are clear evidence of sea erosion. They are definitely wave-cut. The argument lies in the doubt as to the particular level of the sea when the platform was cut.

Our best developed rock platforms near Sydney are at Long Reef, Mona Vale, and Newport. All these are nearly at the same level and they are very definitely intertidal. The average level is, in fact, approximately mean-tide level (say 3 ft. above zero at Fort Denison) so that the sea pours over them well before the time of high-water, even with neap tides and a calm sea. However, the surface is not in one plane. There is often some slope to the outer margin and there are irregularities and even "steps" in the rocky surface so that usually the part of the platform near the cliff may be several feet above low-water level.

One might quite naturally assume that the sea at its present level is still cutting some of these platforms. This is especially true if one judges from the conditions visible at Mona Vale or Long Reef. At the extreme point of Mona Vale, just north of the bay where the baths are situated, the surface of the platform at the base of the cliff is very clean, and there is a freedom from rock fragments which must frequently fall from the cliff. The shale at, and just above, high-water mark is so soft that fragments can be picked out with the finger-nail. One would conclude that weathered material is continually dropping and is being carried away—the rock platform being cut further into the cliff.

Several geologists and physiographers have assumed that these platforms, including some near high-water level, have been cut with the sea at its present level. Others, however, consider that even such intertidal platforms as those named have been cut at a lower level, and their present position represents a rise in land level or what means the same, a general fall in sea-level. Special reference has indeed been made to Long Reef by upholders of this view (Jardine 1925; Jutson 1939). All these platforms are, however, well exposed to rough seas and this makes matters less easy to decide.

At various places on the exposed margins of the rocky coast the rock platforms stand at considerably higher levels than those just described. Obviously,

rock platforms, *with marine remains* at high levels (some have been described on Pacific coasts at heights up to 1,000 ft. and more (see Cotton 1947)) *must* be evidence of a higher stand of the sea. However, all the platforms with which we are concerned are within, or near, present tide levels.

Of the more recent geological works referring to New South Wales rock platforms Jardine (1925) and Jutson (1939) regard the intertidal platforms as exemplified in Long Reef as having been cut by the sea acting under conditions of level such as the present. Others, too, have expressed themselves in support of this for other Pacific intertidal platforms and the view-point is well set out by Jutson. Local geologists, however, favour the other view which is also held by Steers (1929) although he probably only had a brief glimpse of one of the local platforms.

From our own observations we are led to the conclusion that it is characteristic to find one wide rock platform at the foot of the exposed coastal headlands of New South Wales and it ranges from being intertidal to somewhat above high-water mark. These typical platforms whose average height above sea-level varies between narrow limits—usually not more than 6 ft.—give an impression of close similarity. Indeed, a visit on a calm day at the time of low-water might well give the impression that they were all, broadly speaking, at the same level, even though some residues of higher levels are found. We refer now to the rock platforms at Tweed Heads, Yamba, Angowrie, Nambucca Heads, Grant's Head, Norah Head, Boat Harbour, Newport, Mona Vale, Long Reef, Coalcliff, Gerroa, and Ulladulla (Warden's Head). The width of these ranges from approximately 100 ft. (parts of Mona Vale) to 500 ft. (Long Reef and Boat Harbour). From these facts it would be natural to conclude that the platforms named are equivalent, i.e. were formed under similar conditions of sea-level. Now, closer examination reveals unmistakably that the major parts of the different platforms are *not* at the same level. The platforms at Mona Vale and Newport and the greater part of that of Long Reef are not only definitely intertidal, but not higher than mid-tide mark. Indeed, as already noted, there is evidence that the cliff at Mona Vale is being eroded at its base (i.e. at platform level) today, and there is also evidence for similar erosion at Long Reef. A visit to the platforms at Nambucca, Gerroa, and Coalcliff will lead, however, to a very different opinion. This opinion is strengthened by a study of the extensive platforms at Norah Head and much more so by the extraordinary platform at Boat Harbour, near Tuggerah Lakes (the name gives a false impression of the place). All these platforms are wholly, or in part, now definitely above the level which could be the result of wave erosion at present sea-level. We do not want to enter further into the physiographical and geological problems involved.

On the whole we are led to assume that the rock platforms have suffered many vicissitudes since the date of their erosion and that probably most stand at higher levels today. Even those which are now intertidal may have been at higher or lower levels since the date of their first erosion.

The fact that the rock platforms of the coast are not quite at the same level today means more variety for the zoologist and provides an important reason for visiting different places if varied collections are wanted.

SOME NOTES ON THE MAJOR ROCK PLATFORMS WHICH ARE INTERESTING TO THE BIOLOGIST

Almost all of the more extensive rock platforms we have studied present some special features which make them interesting despite the basic similarity in the animal populations, which will be discussed in the chief section of this paper. A brief reference to some selected for special study is desirable. The platforms referred to are taken in order from north to south.

(a) *Nambucca Heads*

The major level of this platform lies just above high-water mark. It differs from all others examined in detail in being composed of strongly folded phyllites—metamorphic rocks believed to be of Ordovician age—and veins of quartz are obvious at a high angle to the horizontal (Voisey 1935). Thus the conditions for erosion are very different from those seen near Sydney where soft shales lie with the layers nearly horizontal. The platform is not exactly an extensive one. Its north-south extension is longest. About 150-200 ft. of the width is fairly level and just above high-water level. From this there is a gradual slope seaward of much broken and eroded rock, the extent of this being approximately 100-200 ft. It is impossible to give a closer figure because of the projecting rocks and eroded gullies. The richer collecting surfaces are in the eroded gullies. Rock pools with sponges and anemones are very noticeable. To the north the platform abuts on a sandy beach and between the two are deep eroded clefts with loose rocks which may be turned over. This is a rich collecting ground. Thus a diversity of habitat is provided. It is noteworthy that there are two large pools at intertidal levels which are paved with Zoanthids. Nowhere else on the long coast of New South Wales have we seen these coelenterates in rock pools or in intertidal regions.

(b) *Norah Head*

There are several platforms at or near Norah Head. That at the headland proper (and just below the lighthouse) is the one which has been most closely studied. The Norah Head platform lies in the Narrabeen Series. The surface dips slightly from a line joining the lighthouse to the extreme tip of the point of the platform. Thus, at the exposed point the height of the platform surface is at least 35 ft. above low-tide level, whilst about 200-250 yards south of this point the platform surface has become intertidal. The platform is fairly wide—approximately 500 ft. from cliff to seaward edge. There is a break in the platform to form a little bay with a sandy beach, approximately 300 yards south of the lighthouse. A path leads down from above at this point.

From what has been said it will be realized that the Norah Head platforms provide many varying conditions for collecting and observation. In fact, this is one of the best collecting grounds on the coast. On the extreme seaward margin at the highest point there are vertical rock surfaces exposed to heavy seas and surf presenting the types characteristic of such surf zones. At the sides of the little bay there are much more sheltered surfaces, and sand and gravel and rock pools. But the feature giving a character to this platform, and seen nowhere else, is the nature of the rock which is largely conglomerate. This seems to have especially favoured the cutting of deep rock pools, many of which are open (by narrow underground passages or cracks) to the sea. In consequence they contain rich growths of kelp and animals of the littoral-sublittoral fringe, and quite large fish may be trapped.

The Norah Head platform will probably turn out to be as rich in species as the famous Long Reef, near Sydney, owing to the diversity of the conditions present. As noted before, Norah Head is a platform which seems to indicate that geological changes in level, etc., have taken place since it was formed by coast erosion.

(c) *Boat Harbour (south of Tuggerah Lakes Entrance)*

In at least two respects this is one of the most extraordinary rock platforms of the New South Wales coast. It is an extensive platform of rocks of the Narra-been Series, practically 500 ft. wide and extending north and south for almost a mile. One of the first points to be noted is that there is no vertical cliff at the landward side. Instead, the high ground rises almost gently from the rock platform and is clothed with grass, shrubs, and trees. There is no doubt that the conditions of tide level here are not those which would favour the erosion of this rock platform today. The surface near the outer margin of the platform is approximately 9 ft. above zero tide level and the rock is sandstone. The land side of the platform is lower and so water tends to collect there. It should be noted, however, that there is little or no headland jutting out where this platform protects the coastline, and the seaward margin of the platform runs nearly north and south. This provides conditions of exposure rather different from those of the long margins bounding bays at Mona Vale, Long Reef, Gerroa, etc.

The fact which makes the Boat Harbour platform extraordinary, however, is the presence of mangroves which are to be found on the land side at the foot of the high ground and facing the ocean. Nowhere else on the exposed seaward face of the New South Wales coast on a rock platform have we seen mangroves. It is impossible at present to offer any exact explanation, except that it is very unusual to find a typical platform such as this without a rocky, near-vertical cliff on the land side. And in most cases any deposition of mud or soil on the platform would be impossible. This is, however, permitted here, and there is a marked seepage of fresh-water at the foot of the hill. The conditions show clearly that there is no erosion on this side. The whole place provides definite evidence in favour of change of sea level, although it may be only a matter of two or three feet.

The result of the presence of mangroves is that very unusual biological groupings exist about them, and here, in close proximity, we find ocean face conditions, together with some of those of estuaries. Side by side one may collect ocean-shore and estuarine species proper to this tide level.

Apart from the above, this rock platform presents another feature which has not been seen anywhere else. The upper surface near the seaward margin bears well-weathered, but distinct, hollows such as those made by the sea urchin, *Helio-cidaris*. Sea urchin holes occur at relatively high levels in some other places where deep permanent pools are found near the platform margin, but those at Boat Harbour are higher still and out of water, well in from the margin, uninhabited and weather worn. Is this a clear proof of a moderately recent change in level?

(d) Newport

The platform at the southern end of Newport Beach is composed of horizontal shales and sandstones of the Narrabeen Series. It is noteworthy as being one of the lowest lying rock platforms on the coast and is covered by the sea until the tide has ebbed to about mid-tide. Even so it is not quite level and the southern part is uncovered long before the northern end is out of the water. Much of the latter lies in the *Galeolaria* zone (see page 196) and the platform surface here provides a very rich collecting ground because of the large number of shallow rock pools with suitably sized stones. There is also a very wide exposure of the lowest tide levels providing one of the best exposures of the kelp zone. All this is partly due to the low level of the rock platform surface, but it is also the result of the configuration of the coast. A detached area of rock platform projects sharply eastward as a very low promontory, in fact, an island, except at very low water. This forms a bastion providing shelter for the shore platform.

(e) Mona Vale

The rock platform referred to under this title is that which extends round the foot of the headland north of the baths. Part of it lies immediately below the extreme tip of the headland. North and south of this the platform curves round (more particularly on the southern side) to form the margin of the adjacent bays.

It is a narrow rock platform, approximately only about 85-100 ft. across from cliff to sea at its narrow end, and 165 ft. wide nearer the Mona Vale sandy beach.

The most interesting places here are at the extreme point, and along the southern margin of the platform which actually runs in a west-east direction and is the first area reached when approached from Mona Vale. Almost the whole surface of the platform which is of chocolate Narrabeen shale is intertidal and it seems to be still extending inward at the extreme eastern point where the cliff is suffering erosion.

By reason of the kind of shelter provided by the direction of the southern margin (east and west) and the presence of submerged reefs in the bay, this platform provides one of the most extensive beds of *Pyura* to be seen near Sydney.

At extreme low-water mark (spring tides) there are glimpses of a secondary platform and the whole is covered with an extensive growth of kelp. This is also an exceptionally good region for the boring sea urchin which is present in thousands in hollows of the rock.

(f) *Long Reef, Collaroy*

This is the best known of all our rock platforms. The geology and physiography have been described by Jardine (1925) and Jutson (1939). Pope (1943) has given an account of the ecology of the best known area, that near the fishermen's settlement on the northern shore.

The platform of Long Reef is a very extensive one. It is found on both the southern and northern sides of the triangular peninsula, and then protrudes seawards from the eastern end for a considerable distance. Actually a rocky reef continues in roughly the same direction but below the surface of the sea and is a recognized danger to shipping. Further description is unnecessary here, except to point out that the rich north-western area is probably one of the most sheltered of all on our ocean coast. This is supported by the fact that small fishing boats are regularly launched from the shore. Another fact of importance is that the extreme eastern point, which is far less frequently examined by naturalists, is exposed and subjected to very different sea conditions from those existing on the sheltered western margin. The zonation is distinctly modified by these sea conditions as discussed on page 197.

(g) *Black Head, Gerroa*

The rock platform at Gerroa round the headland which marks the northern extremity of Shoalhaven Bight is probably one of the most extensive of the southern platforms. There is a very long margin of rock here, and once again the variety of ecological conditions can be emphasized. Most of the rock consists of fossiliferous mudstones of Upper Permian age, and as one traverses the surface one passes numerous fossil Brachiopods.

Most of the platform at Gerroa is just above high-water level and the sea is presumably at a lower level now than when the platform was eroded. The platform at Warden's Head, Ulladulla, stands at approximately the same level and occurs in similar beds. The geology of these platforms has been discussed by Brown (1925, 1928, 1930).

Black Head, itself, is a very exposed promontory and the rock conditions present are also wild looking. Here the animal types which are dependent on a splash zone are found at very high levels indeed. The long stretch of platform from this point south-westward along the bay provides a gradual change, with the introduction of features characteristic of shelter until the limit is reached where the change takes place to sand at the northern end of the Seven Mile Beach.

At this end one notices in particular the repression of *Galeolaria*, the luxuriant nature of the kelp growths, and the great width and obvious nature of the *Chamaesipho* band of barnacles (see Plate 7).

3. TIDES AND PHYSIO-CHEMICAL CONDITIONS OF OCEAN WATER BATHING THE NEW SOUTH WALES COAST

The rocky ocean shores we are considering all face the ocean and are very free from contamination of any kind from the land. There is singularly little effect due to the rivers.

The salinity and the temperature conditions immediately along the shore may be regarded as generally approximating those recorded during several years a few miles out to sea off Sydney. This is suggested on the basis of the turbulent conditions so often existing on the shores and on a few readings which have been made from the rocks.

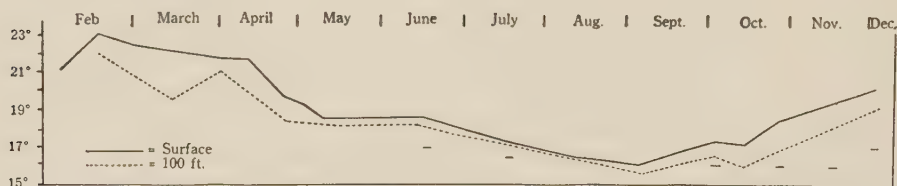


Fig. 2.—Annual range of sea temperatures in °C., from observations made off Sydney by W. J. Dakin.

The graph (Fig. 2) indicates the average range of sea temperatures about four miles off the land in the latitude of Sydney. Through the kindness of Mr. D. Rochford, of the C.S.I.R. Division of Fisheries, we have been able to obtain a few figures for some other places on the coast taken from the research vessel, *Warreen*. From these it would appear that at a station three miles off Cape Byron in the north, the temperature ranged from 20.2°C. in August 1940 to 22.5° in January 1941, and 22.6° in January 1942. The temperatures at our most southerly latitudes are still not known. The striking feature of the Cape Byron temperatures recorded in 1940, 1941, and 1942, is that the *winter* temperature was 20.2°C. compared with temperatures of 13.6° to 16.0°C. which are usual off Sydney.

There has been no organization for prolonged and accurate temperature records at places distant from Sydney on the coast. Nor are there records which will allow close comparisons of rock pool and ocean water. In these there will be much variation during the day, and the height of the pools above low-water level plays a marked part.

Thus, in July 1947, the low temperature of 9°C. was recorded for a rock pool at one of the higher levels of the intertidal zone of the rocky coast. This was the temperature at 8 a.m. and reflected the low night temperatures, but even lower temperatures are probably reached. At the time other rock pools in the vicinity,

but from which the sea had receded not so long before, gave 13°C. as did also the water in a large swimming bath which had just been flooded by the tide.

In the summer the temperature varies in the opposite direction and we find rock pools in the intertidal area with temperatures up to 30.5°C. This is excluding pools high up, but nevertheless filled with salt-water, which are only filled by spray or at high-water spring tides.

The extreme tidal range for Sydney Harbour at Fort Denison is given as 6ft. 7 in. This is a harbour station. Unfortunately, at the present time, accurate information on the tidal range at other parts of New South Wales, and particularly on the sea coast itself, is lacking (see Halligan 1928). But the observations available show that there is little difference, if any, between the northern and southern limits of New South Wales, and the figures given above may be taken, in general, as approximately correct for the open coast.

For many reasons the rock pools present conditions of life which contrast strongly with those of the shores at similar levels. By and large, a rock pool may be regarded as a modified sample of the life of a lower level—perhaps the lowest of tide levels. But even near the lowest tide levels there are some organisms which seem to prefer the changing conditions of the shore and a period, even if short, of aerial exposure, and so are not found in rock pools. A rock pool tends to favour organisms which need permanent submersion. This is particularly well demonstrated by the occurrence of the alga, *Hormosira Banksii* (Turn.) Decne. *Hormosira* covers some rock platforms (e.g. parts of the Long Reef platform, see Plate 2) which, by reason of their level and configuration, remain distinctly wet between successive tides. In other places, however, the only wet intertidal spots are shore pools. Under such circumstances one finds the *Hormosira* always forming a fringe round the margins of the pool, but not on the general rock surface (see Plate 2). It may extend only a very few inches under water; below this it cuts out, and the sides and bottom of the pools, under permanent water, will often be richly covered with other algae such as species of *Corallina*, *Sargassum lophocarpum* J. Ag., *Ecklonia radiata* (Turn.) J. Ag., *Padina pavonia* (Linn.) Lamour, and *Pocockiella* (*Gymnosorus*) *variegatus* (Lamour) J. Ag. (see May 1938, 1939) according to locality or intertidal level.

The higher pools of the shore will be subjected to considerable changes in temperature and light and also in salinity, contrasted with those of the lower levels which are freely filled by the sea.

The highest rock pools filled only by spray or exceptional tides are different again. They are subjected not only to extreme changes of temperature, but to extreme changes in salinity. This is very marked on our coast where long periods without rain may be terminated by great downfalls which flood these pools with fresh-water.

The rock pools differ considerably in nature apart from the difference due to level or distance from margin of platform. One marked difference is to be correlated with their size and depth. On most rock platforms one meets everywhere with little shallow collections of water, perhaps only one inch or two

inches in depth. These are places where certain animals tend to congregate when the tide recedes. The water in such puddles and in slightly bigger ones, must get very hot in summer, or even dry out between tides. The large and deep pools will obviously present a different community. Even in the lowest zones, however, there may be a difference between a pool which is completely cut off from the sea for a period and one which has some deep connection so that there is always contact with the ocean. In these latter one may find the large kelps and fine growths of sponge which are characteristic of the lowest zones and open water.

General Tidal and Wave Effects on Zonation—Splash Zone

It is a well-known and generally recognized fact that the intertidal region of the seashore can be divided into a series of horizontal zones, each of which has its characteristic forms of life. The intertidal animals and plants vary considerably in the requirements necessary for a favourable existence. Some cannot flourish if exposed to the air and sun for more than a very short time and then only on rare occasions. Others prefer to be high up, even reaching places beyond the range of the tide. Between these two types there are many grades and specialities.

There is, however, one particular set of conditions which shows itself more than usually vividly on the New South Wales ocean shore. It is the effect of spray and splash on the exposed coast. Compared with some of the world's coastlines which have been closely studied, the rocky ocean coast of New South Wales would appear to suffer rougher seas for longer periods (see Plate 3). There is no statistical evidence for this. One can, however, not only deduce it from the descriptions of other ecological work, but from our experience at sea, off the coast, during many years of plankton research. The old-time whalers complained of our rough seas. The purse seiners of the Californian coast use fishing techniques regularly which require calm conditions and seem to be of doubtful value here.

This feature is important because the headlands, in particular, present rocky shore contours where, owing to frequent heavy seas, the spray and water splashes keep moist regions which are well above normal high-water mark. Splash zones have been described on other world coasts, but we wish to stress not only the clear effect of splash in pushing some zones to greater heights than usual (and raising intertidal conditions above normal high-water mark), but also the differentiating effects. It is, for example, surprising to find that certain animal associations retain their usual zonation and levels despite the surging water on the wildest sea margins. They demonstrate in the clearest way how some animal species have their shore levels linked physiologically with the tides. On the other hand, some shore forms are affected markedly by the moisture of the spray or water in the condition of foaming seas, and are sensitive to factors which might occur at many

tidal levels. To illustrate what is meant, the barnacle, *Tetraclita purpurascens*, may be taken as an extreme case. It requires moist and shady conditions and is usually found on the shaded surfaces of some vertical or horizontal crack in the rocks. These conditions being given, however, it may be found at almost any level on the seashore from low- to high-tide mark. On the other hand, the ascidian, *Pyura praeputialis*, holds to its very low-water level, scarcely ever being more than 18 inches to two feet above the lowest low-tide mark. The situation becomes still more striking when one observes the barnacle, *Tetraclita rosea*, which loves a vicious lashing by the sea and is therefore generally found near the platform margin. Thus, provided the exposure is sufficient it may actually invade the level characteristic of the Littorinid mollusc, *Melaraphe*, which can exist in the highest zones above tide levels altogether. This latter organism is indeed influenced especially by tide levels, the former by rough wave action and constant splashing or surging.

Keeping in mind these special requirements of intertidal organisms, we shall find that it is possible to select one group of attached animals each of which always keeps to its "proper" levels and thus conveniently serves as an indicator for the zonation of our shores. Fortunately, the most striking of these present this constancy to their particular tide levels, notwithstanding the locality or the varying conditions on the coast. We shall first take these basic or fundamental forms as they appear on a coastal margin, such as that near Black Head, Gerroa, where all zones are present and where the rock slopes at a suitable angle to the lowest of low-tide levels.

We shall compare this with other suitable places and finally refer to positions where intertidal rock platforms are developed at various levels.

GENERAL BIOLOGICAL SECTION

4. THE BASIC ZONATION OF THE NEW SOUTH WALES ROCKY OCEAN COAST*

THE CHIEF ZONES

The northern and southern margins of Black Head, Gerroa, provide excellent localities for an introduction to the study of zonation of our rocky coast. At Black Head a very extensive rock platform is developed, but in general, except where much broken up, its chief and most extensive level stands near high-water mark. Except at its extreme eastern tip, the outer or seaward margins of this platform

* It should be noted that this attempt to formulate a basic zonation for the coast of New South Wales is the result of very many visits extending over a period of years to the more central rock platforms. Numerous visits have also been made to practically every rock platform of any size on the entire coast of New South Wales from Queensland to Victoria. Owing, however, to the great distances and difficulties of travel in some areas, it will be appreciated why the more central parts of the coast have received most attention and the work applies to these in particular.

slope to the water at a convenient angle down to the lowest of spring tide levels where there is visible and readily attainable the very lowest of the intertidal zones.

The conditions met with on this platform can be "checked" at numerous other headlands, and a very good place near Sydney is the headland to the north of Harbord. The main difference between the two places is that the Hawkesbury Sandstone formation exposed at Harbord favours weathering along certain joint planes and so instead of a fairly gradual slope of the outer margin to the lowest tide levels, there are what may be called a series of big steps, and the faces of these steps exposed to the sea are vertical. The same zones are to be seen both at Black Head and Harbord, but the conditions at the former make it easier and safer to examine them, especially the lower tide levels.

At Harbord most of the platform seems to be exposed and curiously subject to heavy surf. Thus the vertical faces we have mentioned receive the full force of the surf, and, in consequence, one finds a highly developed splash zone (see Plates 3 and 7). The same applies at Gerroa, too, in a restricted area near the tip of Black Head, but for the most part the margins of this headland away from the extreme tip are more sheltered and extend a long distance, and clear-cut variations in topography are thus easily available for comparison.

The sequence of associations at these and other localities to be mentioned, remains fundamentally the same despite variations in the degree of exposure. This is one of the most interesting findings. Differences in degree of development of this or that association may and do occur and provide interesting lines of inquiry since the reasons for a poor or good development are by no means obvious.

The animals and plants chosen as key indicators for zonation are few in number. They are the common creatures whose number and position of growth is such as to give character to the shore. In general, they are fixed creatures growing on the exposed rock surfaces, for these are the best for this purpose, although there are some exceptions in the form of one or two species of molluscs such as chitons, limpets, and other gastropods which might almost be called semi-attached for their movements are circumscribed even when the tide is in, and they remain practically fixed when it is out.

The zonation is first referred to tide levels which are determinable fixed marks. We intend to call the intertidal region of the shore the *Littoral*, for that is the usage made famous by the classic work of the earliest shore ecologists and especially by the British naturalist, Edwin Forbes. Accordingly, the littoral belt means the band of shore marked above by the normal high-water line of spring tide in calm water. Its lowest boundary is marked or defined with greater difficulty, especially on the coast of New South Wales where the water is practically never calm. Gislén (1930) takes the low-water mark of neap tide as his

boundary. There seems to be more reason for taking low-water of spring tides and this we would regard as the lower limit. However, this need not be discussed here for we have approached this difficulty in another way which is dealt with below.

The *Sublittoral* region is the area which follows on from the lower boundary of the littoral and is thus normally covered *at all times* by the sea. Its lower limit does not concern us at all since only the upper fringe can be reached from the shore (except by diving).

The *Supralittoral* is the region *above* high-water spring tide levels which has been invaded by only a few typically marine animals, chiefly gastropods. The species are Littorinids, and one might call this the Littorinid zone, the conditions presented being thus similar to those found at this shore level almost all over the world, although in different countries different Littorinid species are found occupying the corresponding places at this level. A point about which to be careful in connection with the supralittoral is the modification of much of this area which may occur when it comes to be near the extreme margin of an exposed coast. In such positions what should be the supralittoral level can be so wet with spray and even with heavy surges of sea-water that it has a special character. The conditions approximate to those of intertidal levels. An interesting feature under such conditions is the presence, not only of some marine forms which are usually associated with lower tide levels, but of a mixture of the typical supralittoral Littorinid types with lower intertidal forms—their occurrence together being determined by the spray, splash, and tides.

The Littoral-Sublittoral Fringe.—The uppermost belt or zone of the sublittoral is very characteristically marked by certain large, "brown" seaweeds (Phaeophyceae). *Phyllospora comosa* (Labill.) Ag. and *Ecklonia radiata* (Turn.) J. Ag. are two of the dominant forms and correspond to the *Laminaria* of other parts of the world's coasts. The line of division between this algal zone and the next zone above is indeed, one of the sharpest "cut-offs" on the shore, and in view of the surging sea this is really noteworthy. The upper part of this algal zone is visible fairly frequently because the large weeds are supported by the water and the upper parts of their fronds often just break the surface.

However, on rare occasions, a zero low tide (or better still, a minus tide) will coincide with a very calm sea. On such occasions it is possible to find *Phyllospora* with intermingled corallines covering extensive areas which are completely exposed. These weeds, therefore, certainly invade the intertidal belt, especially in sheltered places. The photographs (see Plates 3 and 4) were taken on such days. However, under average conditions this zone is normally submerged even at low tides. One might almost say that physiologically it was below the intertidal part of the shore. Yet on calm days it is clearly seen that its upper boundary definitely extends above the mark of low-water spring tides.

Because of all this, and in particular, because a most characteristic animal zone of the shore, marked by the ascidian, *Pyura praeputialis*,* is found at the margin of the littoral and sublittoral areas, and extends not only well above the estimated zero tide level, but also below it, we are calling this strip of shore along the margins of the littoral and sublittoral zones the *Littoral-Sublittoral Fringe*.

This important belt is probably much the same as that described by Stephenson (1939) as the Sublittoral Fringe in his excellent South African work, and by Pope (1943) in her paper on Long Reef. Stephenson states that it is submerged at low-water of *neap* tides and that the sublittoral *proper* (on the west coast of South Africa) is occupied by a rich growth of large algae including a giant species of *Ecklonia*, and *Laminaria*. He also states that in many places the sublittoral fringe is occupied by a giant simple ascidian (*Pyura stolonifera*). All this is remarkably like the Australian conditions on the coast of New South Wales. If one defined the littoral zone as only extending to the low-water mark of *neap* tides, then the term Sublittoral Fringe might have *raison d'être* but Stephenson has not actually defined his Littoral. In view of the uncertainty and difficulty in fixing a rigid boundary at this level, coupled with the fact that the *Pyura* occupies a strip or zone which is definitely partly intertidal and partly subtidal, we prefer the expression Littoral-Sublittoral Fringe—even though it be more cumbersome. This usage is supported by observations on the tide levels occupied by the larger brown algae.

We shall now commence with a description of the lowest shore levels.

(a) *The Littoral-Sublittoral Fringe*

I. *The Kelp Zone*

It is noteworthy that in several places in New South Wales there is a rather level area of rock surface providing an extreme margin to rock platforms and falling exactly in this fringe. In places indeed, it may be called a secondary rock platform at this low level. One sees it at Newport, Mona Vale, Long Reef, Black Head, and Warden's Head, to mention only a few localities.

The lowest zone of the fringe is usually occupied by the large brown algae, *Ecklonia radiata* (Turn.) J. Ag. and *Phyllospora comosa* (Labill.) Ag., the latter only at places south of Grant's Head (lat. 31.30°S.). Near the southern end of the New South Wales coast a bigger kelp, the Bull Kelp, *Sarcophycus potatorum*, joins the *Ecklonia-Phyllospora* association, see page 202. *Ecklonia* is present from

* This common Ascidian has been known in recent years as *Pyura praeputialis* (Heller). There is a possibility that one of the other generic names, *Cynthia* Heller, or *Cynthiopsis* Michaelsen, which have been used for it may have to take the place of *Pyura* Molina and even the long-used specific name may go. But we have kept to the more familiar usage, especially as there is a comparable *Pyura* zone on South African and New Zealand shores, and our species may very well be the same as that of South Africa.

north to south but is poorly developed in the extreme north. Growing between the larger algae are clumps of corallines and possibly some *Sargassum*. Where there is a low level rock platform one may find the ascidian, *Pyura*, growing amidst the algae named, but the closest and thickest growths of *Pyura*, the *Pyura* zone proper, occur immediately above the richer algal layers marked by the big, brown weeds which clearly occupy the lowest zone ever exposed on the New South Wales shores by normal tides.

In some places the *Phyllospora*–*Ecklonia* association shades upwards into low growths of other brown algae—in particular, a stunted *Sargassum* and *Padina pavonia* (Linn.) Lamour. This occurs especially where there is shelter from the surf, and at Mona Vale the succession is *Ecklonia*, *Phyllospora*, stunted *Sargassum*, and *Padina pavonia*, in that order from below upwards.

In places, too, where the growth of *Phyllospora* is luxuriant, odd specimens may be found well above the usual level amidst the *Pyura*. This is still within the fringe. One species of *Corallina* seems to range upwards from the *Phyllospora*–*Ecklonia* horizon, becoming more and more stunted on the way. Whether the same or not, this stunted *Corallina* often forms extensive coverings on the rock platform at mid-tide levels (see page 221).

A much more significant difference in the algal formations of the fringe lies in the nature of the occurrence of the red alga *Pterocladia capillacea* (Gmel.) Born. and Thur. On some of the most exposed headlands, especially if the rock descends vertically or with a very steep slope to depths well below tide levels, the conditions for the growth of the large brown weeds, *Phyllospora* and *Ecklonia*, are less favourable. At such places—and especially where the surf is most violent—the littoral-sublittoral fringe is marked by a growth of *Pterocladia capillacea*. Some *Pyura* may be interspersed with it and here and there the *Pyura* prevails. It is not easy to explain the varying and relative abundance of this red alga and the ascidian at different places. *Pterocladia* is not so sharply limited in its upper growth level as *Pyura*. Like some of the barnacles it flourishes at higher levels but only where there is surf and splash, and for this reason it is not so satisfactory as an indicator of zonation. Its characteristic feature is its love of the pounding surf and the most exposed of all rock surfaces. It does not seem to extend much, if at all, below the zero tide level.

II. The *Pyura* Zone

The characteristic animal of the littoral-sublittoral fringe is, as noted above, the ascidian, *Pyura praeputialis* (Heller). Remarkable growths of this occur at some places and a very definite *Pyura* zone is one of the most characteristic intertidal features on the New South Wales coast. Its upper limit is most regular at a height of approximately 18 to 21 inches above zero tide level. The exact requirements of this creature seem to be fixed firstly, and above all, by the tide level. Neither exposure nor shelter seems to affect this preferred level very much.

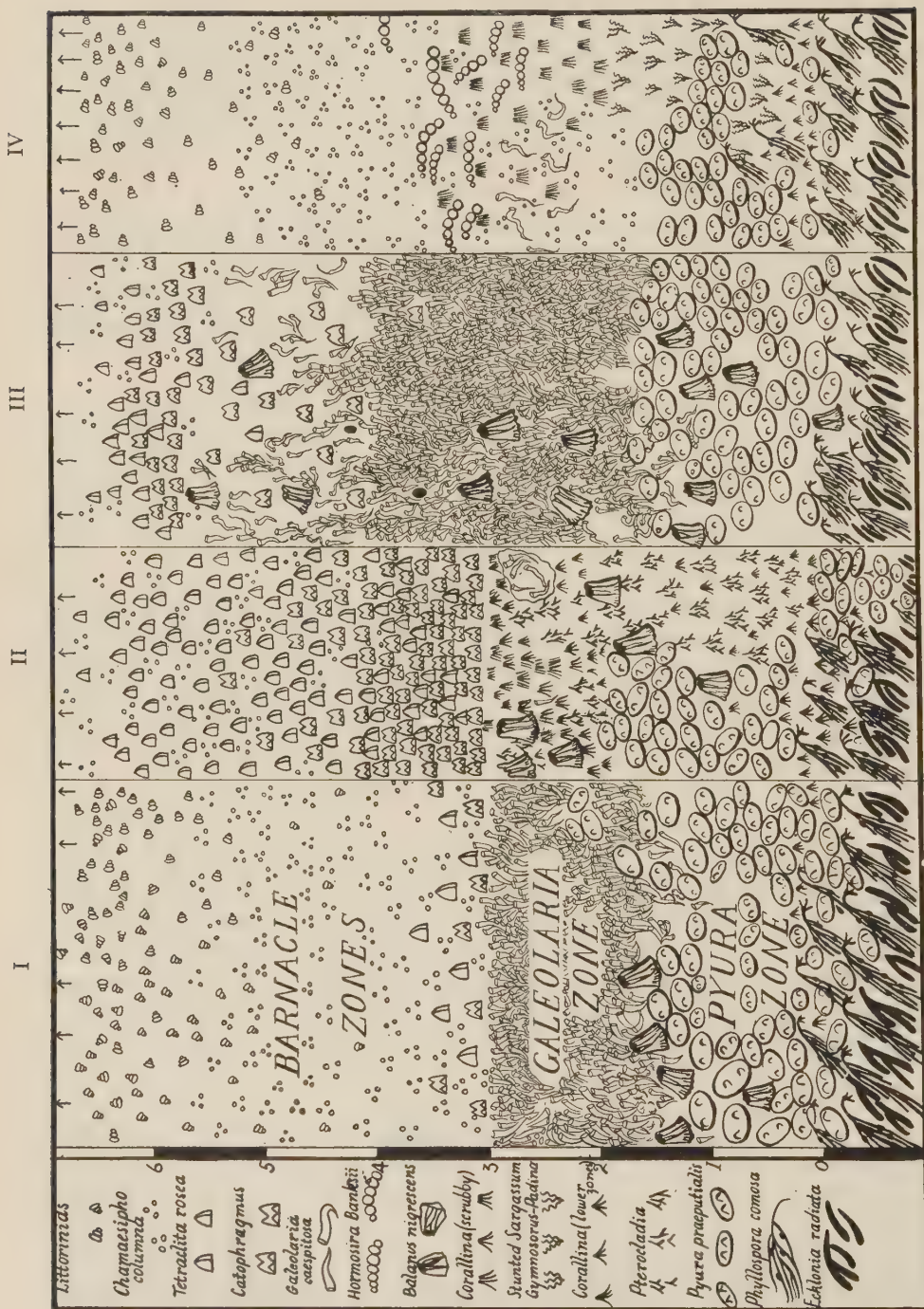


Fig. 3.—See explanation on opposite page.

Apart from the matter of sea-level, it will grow not only where there is considerable disturbance in the water, but also in estuarine entrances (usually, however, opposite the opening). On the whole we would say that it likes rough ocean water on the open coast. Some of the finest growths we have seen occur on level surfaces just sheltered from the fiercest sea attack. This can be seen immediately north and south of some of the headlands along the sides of the bays, Mona Vale and Warden's Head providing two excellent samples.

The photographs (see Plates 4 and 5) illustrate these remarkable growths of *Pyura* on our coast. It is not surprising that this animal obtained a native name, Cunjevoi, shortened to Cungy by the rock fishermen who have destroyed tons of it by using it as bait.

An interesting feature about this ascidian is that it is represented by an almost identical form growing at the same level and forming similar encrustations on the seashores of both New Zealand and South Africa. Oliver (1923), for New Zealand, states that it is dominant "in a few localities." He states that he has not been able to find the name of the species and it is to be noted that he gives its height as 6-8 cm. which is much smaller than our *Pyura*.

The South African species is given as *Pyura stolonifera* and is also described as a type marking the lowest intertidal levels. Stephenson states that the *Pyura* community reaches its apotheosis on the south coast of South Africa. "On a very large number of exposed reefs in the fringe" (our littoral-sublittoral fringe) "this leathery ascidian conceals the rock in incredible numbers, closely packed; it must cover, altogether, many inches of rock." This resemblance to our condition is exceptionally interesting. We can add, however, that the Australian species seems to occupy even a greater length of coastline. On the East Australian coast alone, it is found along 1,000 miles of coast, between the parallels of latitude 28°S. and 38°S. So far as New South Wales is concerned it is impossible to say whether it is less developed in either the north or the south after examining the rich beds at Yamba in the north and Eden in the south. Harvey Johnston (1917) records it at Caloundra, Queensland, although he states that it is not so common there as at Tweed Heads on the New South Wales border.

Explanation of Fig. 3.—Basic zonation of shore and some variations. The scale is approximate and only applies to levels measured vertically above zero tide mark. Vertical measurements are given in feet, zero being the low-water spring tide level at Fort Denison, Sydney Harbour.

Column I. Average conditions facing ocean, reasonable shelter. Typical basic type.

Column II. Very exposed ocean face, swell reaching near vertical or vertical face of rock.

Column III. More exceptional conditions with considerable exposure but with reefs breaking the swell. (Long Reef, extreme point.)

Column IV. Rock platform and margin in very sheltered locality, and often near sandy beach, e.g. rock platform at Black Head at westward limit, and Norah Head.

In Column II the surf barnacles are greatly developed and the barnacle zones extend high above normal limits (splash zones). In III the *Galeolaria* zone is extended upwards and continued by barnacles. In IV the *Galeolaria* is poorly developed and *Chamaesipho* extends in a wide band between the *Pyura* and the supralittoral, or the *Galeolaria* is replaced by algae (extreme right).

The *Pyura* association shelters a number of different animal species. These are listed in the next section which deals in greater detail with some of the shore associations. Attention might be drawn here to the moderately large sea urchin, *Heliocidaris erythrogramma*, which in places occupies rounded hollows in the rock surfaces on which the *Pyura* grows and may almost honeycomb it. It should also be noted that in certain exposed places a very large rock barnacle, *Balanus nigrescens*, occurs not only amidst the *Cunjevoi*, but above it.

Further details of its occurrence are, however, given later for its presence evidently demands splash. At the top of its range it cannot be said to be abundant and even where there is splash its occurrence is often rather capricious.

(b) *The Mid- and Upper-littoral*

III. *The Galeolaria Zone*

Immediately above the *Pyura* zone, and therefore beginning just about the upper margin of the littoral-sublittoral fringe, is an association of an entirely different kind. This is the *Galeolaria* association. It is as remarkable as *Pyura* in the sharpness of its limits, but this sharpness must be coupled with a certain important proviso concerning the nature of its growth, as follows.

Galeolaria caespitosa Lam., a polychaete worm living in a secreted tube of lime, may be found alive as isolated individuals well separated from one another, at almost any intertidal level on the shore, provided the spot is sufficiently moist, but *Galeolaria* also occurs in a very different condition as a very crowded encrustation in which masses of tubes are closely packed and intertwined. In places such a crust may reach a thickness of eight inches and look like coral. In fact, growing in this way it is often popularly called "Sydney coral." This is the condition in which *Galeolaria* forms a highly characteristic belt along our shores, and even where only an inch or less thick this crowded encrusting state can be easily recognized as a clear-cut shore feature (see Plates 5 and 6).

Galeolaria in the form of this close-growing encrustation occupies a sharply limited zone just above the *Pyura*. On a vertical face the width of this zone as a band is usually only about 18 inches and at most two feet. Its upper margin is, in fact, at about the three-foot tide level. On a suitable slope the width will naturally be greater. At Harbord and above all at Merewether, south of Newcastle, and at other places, rock platforms may be found the heights of which just coincide with the *Galeolaria* level. At these places very extensive growths indeed may be found and many square feet of the rock platform will be covered, the growth being especially thick on the corners and edges of rocks (see Plate 5).

The sharp vertical limit of the *Galeolaria* is well illustrated in the photograph of the conditions at Black Head, Gerroa (see Plate 6). The existence of such a margin at a place where the sea breaks violently for most of the time is a very remarkable phenomenon and one almost impossible of adequate explanation at present.

It will be seen that *Galeolaria* in its thick, crusty form occupies the middle zone of the intertidal region. On a vertical face there will roughly be only six feet marking the total rise and fall of exceptional spring tides. The lowest limit of the *Galeolaria* encrusting zone may be approximately about 18 inches to two feet above tidal zero mark of Fort Denison and the upper limit as stated comes near the three-foot tide level.

The upper limit is extraordinarily regular in its occurrence and in most places varying conditions of exposure and shade will scarcely shift it more than an inch or two.

But in very exceptional places this limitation of the *Galeolaria* to a belt 18 inches to two feet wide is changed. We have illustrated such a condition in column III of our typical zonation in the diagram (Fig. 3). It is not easy to define the conditions which bring this about. Our best example is a very restricted area on the extreme tip of Long Reef. Now this is an exceedingly exposed point and as a rule extreme exposure restricts *Galeolaria*. There is, however, one feature of note at this locality. The vertical faces of the rock platform (average height at this point 10-12 ft. above zero tide level) do not pass down to deep water, but to a considerable area of reef or secondary platform which is at about zero tide level. Thus we have a very exposed coast where, however, the swell is broken at periods round low-water by a reef of considerable extent. Thus, broken surf must reach the *Galeolaria* face where ordinarily, in such a position, solid waves would break against it. Here the *Galeolaria* crust extends up to a height of approximately 5 ft. 6 in. above zero tide level. Similar vertical extensions have been noted elsewhere where the same conditions exist.

In the *Galeolaria* band the close growth of limy worm tubes seems not altogether favourable as a surface for creeping sedentary univalves such as limpets etc. (which enjoy the spaces between *Pyura*) and at first sight it would appear as if nothing else but *Galeolaria* occurred. (This is not the case at Nambucca Heads where in places the *Galeolaria* surface is dotted over by the chiton, *Liolophura gaimardi*.) Careful examination of the crust shows, however, that this is very far from the truth and that actually *in* the mass, and *between* the tubes, there is a rich association of small animals, five species at least of which are so closely linked up with the *Galeolaria* (and in many ways noteworthy) as to require special mention.

They are the barnacle, *Ibla quadrivalvis*, the pulmonate, *Onchidium patelloides*, the little bivalve, *Lasaea australis*, an unknown species of a polyclad or turbellarian, and, strangest of all, a spider, *Desis crosslandi*.

A number of other organisms are found in interesting ecological relationship with *Galeolaria*. Reference will be made to all these, with *Galeolaria*, in the next section, where further details of the associations are given.

The *Galeolaria* zone has been noted as far north as Moreton Bay and we have followed it south to the Victorian border where it is still very much in evidence.

Formations of tube-worms have been recorded from other of the world's coasts at about the same level as that at which the *Galeolaria* occurs here, but this characteristic and common member of the family Serpulidae does not seem to be represented in this particular way by any close ally on the South African shores, although in places, at about the same level, there are great expanses of the sandy tubes of another polychaete genus (*Gunnarea*). And the shore of New Zealand is not very different, for Oliver (1923) specifically mentions that the *Galeolaria caespitosa* association of Australia might well be regarded as represented on New Zealand shores by the sandy worm tubes of *Hermella*. There is a tube-worm related to *Galeolaria* forming masses of calcareous tubes in restricted localities on the New Zealand shores (and at about the same tide level) but it is in the muddy waters of Auckland Harbour. This is the worm, *Vermilia carinifera*. It seems to be very much larger than *G. caespitosa* (3.5 cm. in length) and the tubes larger still.

Up to this point we have been dealing with the lower and middle zones of the shore and this has, of necessity, forced our attention to positions on or very close to the outermost margins of the coast and rock platforms. The next zones upwards may present different appearances here and there in consequence of the varying possibilities in the nature of exposure of this part of the shore. The variations are, however, clear and simple, and largely concerned with one animal group—that of the barnacles. Several species are characteristic indicator types of the zones immediately above the *Galeolaria*.

IV. The Barnacle Zones

The upper zones of the intertidal region of the coast are typically barnacle zones, although the greatest development of these lies immediately above the *Galeolaria*.

It is essential at the outset to stress again two possible types of locality (the tide level being the same), the exposed locality on the outer tips of headlands with splashing surf and the more sheltered parts of headlands which usually face north or south.

On sheltered margins the *Galeolaria* is usually followed upwards by a zone (often a wide one if the shore presents a gentle slope) marked by a small barnacle, *Chamaesipho columna*. This is, in fact, the smallest of our barnacles of the ocean rocky shore. It may cover the rocks with a close mat in which one individual touches others on all sides. We have counted approximately 3,000 in a square foot and there might easily be more. It is one of the characteristic South-West Pacific animal species and is found on New Zealand shores in a similar position. *Chamaesipho* seems to fade out along the extreme northern part of the coast, north of or about Ballina, and we have information that it is missing in southern Queensland. Except for this change *Chamaesipho* is characteristic of the New South Wales coastline.

A very true and exceptionally clear-cut picture of the general position of *Chamaesipho columna* is exhibited by the rocks on the south side of Black Head forming the northern end of Shoalhaven Bight. Here the *Chamaesipho* forms a whitish band which may be 30 ft. wide owing to the gentle slope, and which can be seen from the cliffs as it extends along the shore for a mile or more (see Plate 7). Near the village of Gerroa the *Galeolaria* zone is practically missing and its place is taken by *Chamaesipho*. Where the rock rises almost vertically from the Cunjevoi zone one can measure the barnacle's vertical extension. At one place this reached approximately five feet above low-water mark, that is, nearly to high-water limits. There are other localities which are unfavourable for *Galeolaria*, and the *Chamaesipho* may be seen invading this zone. Apart from the disappearance noted in the extreme north, *Chamaesipho* is probably the ubiquitous fixed animal of our rocky shores for the platform surfaces often lie somewhere in this zone, and although at exposed places, the lower part of the zone is taken over and occupied by the surf barnacles next to be mentioned, closer examination will probably show *Chamaesipho* amongst them.

On the coasts where an intertidal rock platform is developed at a suitable level, as at Long Reef and especially at Mona Vale, the width of the surface on which *Chamaesipho* is well established becomes much greater. The species is obviously capable of withstanding considerable variations in the duration of exposure and submergence. Typically the zone may be stated as approximately that between high- and low-water mark of neap tides.

On surfaces where the *Chamaesipho* is less crowded one meets with the limpet, *Cellana*, and other gastropods. Within its geographical range *Sypharochiton* also occurs here. The common associated forms are, however, listed in the following section.

At high levels where the upper boundary of *Chamaesipho* is approached one often finds another barnacle species intermingled with it. This is *Chthamalus antennatus*. At the highest intertidal levels the barnacle zone is represented exclusively by this latter species and in places it develops into a close barnacle community in which each specimen touches its neighbours. Such a condition may be seen at Yamba on the far north coast, or at Harbord, Newport, and many other places, usually in restricted areas. The growth of *Chthamalus* is favoured by spray, but its occurrence is such as to make it a valuable indicator type for zoning purposes.

We have taken *Chamaesipho columna* and *Chthamalus antennatus* first because their positions on the shore are distinctly indicative of certain tide levels which scarcely applies to the three rather more "exciting" barnacle species to which we now draw attention.

At places where the ocean surf beats with all its force, the lowest part of the barnacle zone is occupied by a combined association of *Tetraclita rosea* and *Catophragmus polymerus*. *Catophragmus* is actually known locally as the "surf

barnacle," but it is not easy to see why *Tetraclita rosea* should not receive the same appellation since it, too, loves the break of heavy surf.

The association of the two barnacles is, however, not one of two equal partners. Usually just above the *Galeolaria* zone (and in those places where there is danger to life unless the sea is very calm) one finds a closely packed crust of these two largish barnacle species. *Catophragmus* is probably always the more abundant here, if conditions are sufficiently wild, so much so, that one may at first be tempted to think that only this species is present. Closer examination will probably reveal some *Tetraclita rosea*. This thick, mixed band may extend vertically for about three feet where there is a vertical face on which waves break (and it is only in such vertical or near vertical places on a very exposed margin that one will find the best closely packed "crusts," more especially a crowding of *Catophragmus* which prefers a vertical surface). Above this the *Catophragmus* quickly become fewer and fewer in relation to the *Tetraclita rosea*, and at the same time *Chamaesipho* begins to appear.

Tetraclita rosea may, however, form a close community for several feet more. (Note: This obviously brings it above high-water mark, but in those places where *T. rosea* is found in numbers, we are really faced with physiological rather than actual tide levels, and the conditions normal to lower zones may be pushed high up the cliffs.) Since the rise and fall of the tide on the New South Wales coast averages less than five feet it will, of course, be realized from the above that the presence of *T. rosea* and *Catophragmus polymerus* is not a matter of tide levels at all.

T. rosea is a moderately large, rough-looking barnacle with a characteristic pink colour, and when rock surfaces are crowded with this species the colouring effect is very conspicuous.

One might almost say that any place where these two barnacles occur can be a dangerous position for rock fishermen, meaning that, with a rising tide and rough sea, they are always within the reach of breaking waves.

The sea coast at Harbord presents an exceptionally favoured place for the examination of *Tetraclita rosea* and *Catophragmus polymerus* because it is a particularly exposed headland and in places there are inclined rock surfaces up which the waves run easily. Here we have found *T. rosea* at a height of nine feet above low-water level, and, at one place, as much as 68 ft. horizontally inwards from the outer margin of the shore (see Plates 7 and 8). With regard to the geographical range of these two species similar fine growths can be found in the north at Cape Byron and also at Point Danger on the Queensland border. *Catophragmus* has also been recorded by us up to Moreton Bay. Both species are still present at the southern end of our coast, but it should be noted that *T. rosea* is not nearly so common in the south (see page 204).

Thus, to sum up, one finds the sequence, *Galeolaria*, *Catophragmus-Tetraclita*, *Tetraclita*, *Chamaesipho-Chthamalus* (passing from low to higher levels) in

fairly exposed places, and *Galeolaria*, *Chamaesipho*, *Chthamalus* on more sheltered rock shore margins. Exceptionally, the *Galeolaria* may be missing altogether.

Before passing to the supralittoral it is necessary to mention a more restricted association which in certain limited localities may also occupy the surface of the rock in close numbers. It is a limpet community, but is almost as definite as that of the barnacles for the members are certainly fixed when the tide is out, and they probably only move a few inches (and return) when covered, which will only be at spring tides. The species is *Notoacmaea petterdi* and where found in a crowded condition its occurrence is characteristic. The requirements for this seem to be (1) a vertical surface, (2) a high level near the upper limits of the intertidal zone, and (3) a certain amount of moisture in the air which implies a position not too far horizontally from the outer edge of a rock platform. Its tide level is then as indicated above, at the uppermost margin of the littoral zone (indeed, in special places it would appear to be well above all tide levels).

Despite what is stated above, *Notoacmaea* may also occur on non-vertical, even horizontal surfaces, and with the greatest diversity at almost all levels. But where the above conditions are present one may find definite bands of *Notoacmaea* and we recorded them at Yamba in the north, at many places in the latitude of Sydney, and in the far south of the New South Wales coast at Eden.

(c) *The Supralittoral*

V. *The Littorinid Zone*

The highest zones of the rocky ocean shore, and we refer here to those well above all tide levels, are naturally not richly frequented by species of marine life. One might add that this should not be surprising since the surface of the dry rock reaches very high temperatures under our blazing summer sun. Under these circumstances it is very interesting to find that two species of *Gastropoda* are numerous in this zone. They are *Melaraphe unifasciata* and *Nodilittorina tuberculata*. *Nodilittorina* is found higher than *Melaraphe* (in fact it has been recorded from 40 ft. above sea-level). It is the characteristic species of the supralittoral. *Melaraphe unifasciata* also extends downwards into the intertidal zones and juvenile forms are frequently found together with the barnacle, *Chamaesipho*, and even down with *Pyura praeputialis*.

The little *Melaraphe* is interesting in that it illustrates, in conjunction with the barnacle, *Tetraclita rosea*, two different types of reaction to tide and splash. As we have noted, *Melaraphe* is adapted to the supralittoral levels where it may be seen exposed for long periods to a hot sun and dry air. *T. rosea* likes rough sea water and much splash. On exposed headlands where waves break higher than the mid-tidal zone for much of the year one may meet *Melaraphe* and *T. rosea* side by side under the wet conditions favourable, indeed essential, to *T. rosea*.

5. THE GEOGRAPHICAL RANGE OF THE BASIC ASSOCIATIONS

It has been stated in the previous section that very little geographical change occurs in the basic zonation of the rocky ocean shore between the Queensland and Victorian boundaries.*

Differences no doubt occur in the presence or abundance of some of the many shore species which have been collected in this long range. Quite a number of such have been noted, but they are amongst the less common species. Probably many are still to be discovered. To establish certainty of real presences or absences of the rarer forms would require very extensive collecting indeed at different localities and at all times of the year in each locality, for we have found that many animals of the rock platforms are seasonal and migrate.

There are, however, about half a dozen striking changes in the range of the common members of the basic associations and these deserve very special mention, especially since the number is so small. The species concerned are:

Algae

Phyllospora comosa
(a large kelp-zone species)

Sarcophycus potatorum (Bull Kelp)

Mollusca

Loricata—

Sypharochiton septentriones
Liolophura gaimardi
Onithochiton quercinus

Gastropoda—

Cominella alveolata

Pelecypoda—

Brachydontes rostratus

Crustacea

Chamaesipho columna

Tetraclita rosea

Phyllospora comosa (Labill.) Ag.—This alga, as will have been noted, is described as forming, with *Ecklonia*, a very distinctive and sharply marked association in the lowest intertidal zone and below it. These large brown weeds are equivalent to the *Laminaria* of rocky shores of other parts of the world. It is all the more significant to notice, therefore, that *Phyllospora* “cuts out” rather sharply somewhere in the neighbourhood of Grant’s Head (lat. 31°30’S.). Nowhere north of this do we find the characteristic pastures of *Phyllospora* which are so obvious in the south.

Sarcophycus potatorum (Labill.) Kuetz.—This is almost as noteworthy as *Phyllospora* in its occurrence, except that it is much more restricted “in mileage” along our coast. This is one of the giant kelps. It is a southern form and occurs in the lowest zone characterized by *Phyllospora* and *Ecklonia*. It “cuts out,”

* A reference to Johnston (1917) will show that many animal species listed by us are present at Caloundra, Queensland.

however, north of Bermagui (lat. $36^{\circ}30'S.$) and is only found south of that latitude. The presence of this large seaweed on our extreme southern coast is quite a feature in the seascape at low-water spring tides.

Sypharochiton septentriones (Ashby).—This is one of the three chiton species which live clearly exposed to view on the rock surfaces (albeit in small moist depressions or cracks). It is found over a rather wide band of shore from the *Galeolaria* zone upwards so long as slight moisture is available. (Perfectly sculptured juveniles appear amongst the *Pyura*.) Somewhere about the latitude of Grant's Head or Trial Bay, *Sypharochiton septentriones* "cuts out," but curiously enough its place is taken by a species of a different genus, *Liolophura gaimardi* (Blainville), which occurs from that point northwards and then along the Queensland coast.* This latter species is probably more usually found in the lower parts of the *Chamaesipho* zone or even in the *Galeolaria* zone. (It was very common in the *Galeolaria* zone at Nambucca Heads.)

Strangely enough *Sypharochiton* also disappears again in the extreme south, and certainly is not common south of Bermagui. As an easily seen and common shore creature its geographical range is thus limited to the central parts of the coast. Of the two other chiton species of the exposed rock surfaces, *Poneroplax paeteliana* (Thiele) is plentiful along the entire coast, but *Onithochiton quercinus* (Gould) is absent from the extreme south, its range ending between Narooma and Bermagui. It inhabits the lower zones of the shore and is almost always found below the *Galeolaria*.

Brachydontes rostratus Dunker.—This mussel is clearly a southern form and it occurs in very large numbers covering the shore in "sheets," on parts of the Victorian coast. In southern New South Wales it is restricted to cracks in the rocks where considerable numbers may be found crowded together.

It has not been found by us north of Tuross Heads (lat. $36^{\circ}10'S.$), but occurs from there southwards.

Cominella alveolata Kien.—This is a gastropod of the family Buccinidae and the species is apparently absent from the entire stretch of the New South Wales coast except the extreme southern end. Thus it plays little or no part in our general zonation. But it suddenly turns up in numbers at Twofold Bay and is an interesting case since the species is known along the southern coast of Australia and is common at Kangaroo Island, near Adelaide.

C. alveolata is found at about the same level as that of the gastropod *Morula marginalba*, in the *Chamaesipho* zone of the shore. Both are present at Twofold Bay.

* This statement applies only to the open ocean coast.

Chamaesipho columna.—The disappearance of this small barnacle in the extreme north of New South Wales is perhaps the most striking change amongst the zonation indicators. Everywhere else it is a very common and characteristic feature of the upper half of the littoral (see page 198).

Tetraclita rosea Darwin.—This surf barnacle which plays the prominent part described in the text along most of the New South Wales coast, becomes considerably reduced in numbers in the extreme south, so that whilst present in the same situations in conjunction with *Catophragmus*, its partnership in this association (see page 200) is very seriously reduced.

It would be legitimate to refer the geographical boundaries of all the above species to sea temperature changes along the coast. As already noted, the change is gradual and the number of common species affected is very small.

6. THE COMMON ANIMALS ASSOCIATED WITH THE BASIC ZONES OF THE NEW SOUTH WALES ROCKY OCEAN SHORE

In this section we shall list the common shore animals which are found more or less closely linked with the associations previously enumerated and where necessary further notes in regard to the biology of selected species are added. A brief reference only is made to the more important algae.

It should be obvious that the greatest number of species, and, indeed, the greatest number of individuals in the different shore zones, tend to be found where, owing to the occurrence of nearly horizontal platforms or gentle declivities, the zones, and more especially the lower zones, cover a greater area. Horizontal platforms provide every kind of niche beloved by the shore animals for, owing to cracks and hollows, they provide pools of all sorts and sizes, shallow and deep, together with overhanging ledges, stones, and overhanging rocks with beautifully sheltered holes and crannies beneath them. Even a rough weathered surface provides useful depressions which retain sea-water, or provide shade, or both.

We shall follow the same sequence as in the preceding section and commence with the lowest region taking first a platform in the littoral-sublittoral fringe.

(a) *The Littoral-Sublittoral Fringe*

A warning might be given here in regard to the littoral-sublittoral fringe to the effect that our lists apply mainly to places just away from the most exposed points of the coast. One can see the algae, *Pterocladia*, *Ecklonia*, and the coral-lines, and the ascidian, *Pyura*, at such points at low spring tides on calm days, but real collecting at these exposed places is rarely possible. The necessary protection to allow of collecting may be provided by a detached rock acting as a breakwater, or a mere bend or break in the platform margins.

I. The Kelp Zone

The littoral-sublittoral fringe is characterized, as we have seen, by the large brown weeds, *Phyllospora comosa* (Labill.) Ag. and *Ecklonia radiata* (Turn.) J. Ag. (especially at the lowest levels), together with corallines and smaller brown weeds. Owing to the absence of *Phyllospora* north of Grant's Head and the gradual fading out of *Ecklonia* in the extreme north, the conditions of the fringe are different north of Byron Bay. The fringe is then characterized by the abundance of *Corallina*.

In the most exposed places of all, and usually where the platform presents a vertical face to the ocean, the red alga, *Pterocladia capillacea* (Gmel.) Born. and Thur., may be abundant.

In some localities there may be platforms of softer or deeply eroded rock with countless semi-spherical hollows each containing a large specimen of the echinoderm, *Heliocidaris erythrogramma* (see Plate 8). Exactly similar formations have been found in widely distributed and distant parts of the world but with different sea urchin species burrowing in the rock and apparently making the holes (in which they fit fairly closely) by means of their spines or teeth or both. *H. erythrogramma* occurs from Queensland to South and Western Australia. In places in this zone one also finds odd specimens of a related species, *H. tuberculata*. A few may always be found at Mona Vale in the summer but for some reason or other they were far more numerous at South-West Rocks, Trial Bay, in September 1946 than anywhere else. Empty sea urchin holes are sometimes lined with thin layers of an algal nature, often with cushions of a green, velvet-like *Codium*, or the calcareous encrusting algae, *Lithothamnion* or *Melobesia*. They become shelters for small anemones and sponges (the latter being species which we have not been able to identify as yet). Between the sea urchin holes or the *Pyura* clumps and at a level above that preferred by the largest brown weeds, there will probably be masses of corallines (*Corallina* spp. and *Amphiroa anceps* (Lamk.) Decne.) and the rock surface itself is typically pink in colour with encrusting calcareous algae.

II. The *Pyura* Zone

The *Pyura* association needs particular attention. It usually attains a height of 18 inches to two feet above zero low-tide level. *Pyura* has been stated by Hedley to demand rough seas. This is not quite correct, but it is not altogether simple to define the limiting conditions. *Pyura* likes unadulterated ocean water for its best development and a considerable amount of movement in the water is desirable, but not an extreme amount. In fact, in the most exposed places *Pyura* does not extend upwards so far as in more sheltered localities. (An altogether exceptional height is nearly 4 ft. above zero tide level. This was seen in a few places in Twofold Bay at the extreme southern end of the New South Wales coast.) It is also found in the estuaries and almost land-locked bays like Jervis Bay, but usually in such places it is best developed where it faces the estuarine opening to the ocean.

COMMON ANIMALS CLOSELY ASSOCIATED WITH PYURA

Porifera

Purple and orange encrusting sponges—
probably a *Haliclona* sp.

Coelenterata

Hydroida—

Sertularia elongata Lamx.
S. operculata Linn.

Worms

Polychaeta—

Galeolaria caespitosa Lamarck
Salmacina australis Haswell
Lepidonotus argus (Qtrfg.)
Lysidice collaris Grube
Nereis zonata Malmgren var. *pessica*
Fauvel

Pseudonereis masalacensis Fauvel
Perinereis novae-hollandiae Kinberg

Nemertinea—

Small, greenish-grey nemertian (unidentified), very common

Crustacea

Cirripedia—

Balanus imperator Darwin
B. nigrescens Lamarck
B. algicola Pilsbry
Elminius simplex Darwin
Tetraclita rosea Darwin
T. purpurascens (Wood)

Amphipoda—

Leucothoe commensalis Haswell
(inside *Pyura*) and other unidentified species

Tanaidacea—

Unidentified sp. (very common)

Brachyura—

Pilumnus vestitus Haswell

Mollusca

Loricata—

Poneroplax paeteliana (Thiele)
Onithochiton quercinus (Gould)
(except in extreme south where it is missing)
Craspedoplax variabilis var. *cambrica*
Ired. & Hull
Meturoplax retrojecta (Pilsbry)

Gastropoda—

Patelloidea alticostata Angas
Montfortula conoidea (Reeve)
Dicathais orbita (Gmel.)
Pelecypoda—
Lasaea australis Lam.

Echinodermata

Echinoidea—

Heliocidaris erythrogramma (Val.)

Centrostephanus rodgersii (Agassiz)

Chordata

Ascidacea—

Amaroucium sp.

Boltenia (Pyura) pachydermatina
Herdman

The very nature of *Pyura praeputialis* with its strong, rough, and leathery coat favours the attachment of a number of organisms. Still others seek the shelter found between adjacent individuals. One expects, therefore, to find an association of species, adapted to the physio-chemical conditions as well as to

each other. In addition to the following list of animals, the algae *Ulva lactuca* Linn., *Colpomenia sinuosa* (Roth.) Derb. and Sol., *Dictyota dichotoma* (Huds.) Lamour, *Pocockiella* (*Gymnosorus*) *variegatus* (Lamour) J. Ag., and a short reddish alga which covers the whole surface, are found adhering to the tests of the *Pyura*.

Often found crawling over and among the tests of *Pyura* is the smallish slit limpet, *Montfortula conoidea*. This shell is found typically in rather moist sheltered situations where it browses on the algae. It is not, however, confined to the littoral-sublittoral fringe alone, being found in higher zones, especially in rock pools and crevices and among the short, tufty, stunted *Corallina* of the mid- and upper-littoral parts of the reef.

We have shown that it is possible to distinguish two roughly marked zones in the littoral-sublittoral fringe. Lowest of all is that characterized by the big brown weeds (with *Pyura*, *Heliocidaris*, and an undergrowth of the smaller red weeds making up the common types to a greater or lesser degree according to amount of exposure and nature of rock). Above this zone we still find that the *Pyura*, *Heliocidaris*, and the corallines are continued with an admixture of the smaller brown weeds such as *Sargassum* spp., *Padina pavonia* (Linn.) Lamour, and *Colpomenia sinuosa* (Roth.) Derb. and Sol.

The very large barnacle, *Balanus nigrescens*, occurs in groups or as scattered, isolated specimens in both the kelp and *Pyura* zones. It is best observed, and reaches its largest size, in the *Pyura* zone and even above this.

On an exposed part of the ocean coast at Nambucca Heads, the algal zoning in the sublittoral fringe was as follows: *Ecklonia*,[‡] *Ecklonia* interspersed with brilliantly red corallines, then *Sargassum*, then stunted *Sargassum*. Next came a low scrubby coralline encrustation, the branches of which feel gritty and come away in particles. It is a very common condition of the higher growths of *Corallina*. This algal sequence is very characteristic of the outer margins of the Long Reef platform and many others. Above the sublittoral fringe the alga, *Hormosira Banksii* (Turn.) Decne. appeared. The *Galeolaria* zone came approximately between the stunted *Sargassum* level and the *Hormosira* level.

LIST OF COMMON ANIMAL SPECIES OF THE LITTORAL-SUBLITTORAL FRINGE

(Additional to those of the *Pyura* Association mentioned above†)

Those marked with an asterisk occur also in higher zones.

Porifera

Tethya (*Donatia*) *corticata* Lend.

(There are also about six common encrusting sponges in this zone which have not been identifiable as yet)

[‡] *Phyllospora* is, of course, absent at this latitude.

[†] It will be obvious that each species occupies its own characteristic habitat on the platforms. Some only occur where there are collections of sand or gravel, others are under stones, in pools, in crevices, or on the bare rock.

Coelenterata

Hydroida—

- Halocordyle disticha* var. *australis* (Pennaria *australis*)
Myriothele australis Briggs
Tubularia gracilis V. Lend.
Obelia australis V. Lend.
Silicularia campanularia V. Lend.
Sertularia spp.
Plumularia spp.
Aglaophenia spp.

Madreporaria—

- Cylicia quinaria* Tenn. Woods

Gephyrea—

- Physcosoma japonica* (Grube)*

Polychaeta—

- Sabellastarte indica* Sav.*
Identhyrsus pennatus (Peters)*
Spirorbis sp.
Galeolaria hystrix Mörch.*
Diopatra dentata Kinberg*
 (limited to places where sand is present in cracks of the rock platforms)
Eunice aphroditois (Pallas)
Nereis pelagica Linn. loc. var.

Retepora sp.

Beania magellanica Busk

Membranipora membranacea Linn.

Loricata—

- Ischnoradsia australis* (Sowerby)*
Cryptoplax mystica Ired. & Hull
Haploplax smaragdina (Angas)*
Callistelasma antiqua (Reeve)*
Rhyssoplax jugosa (Gould)*

Gastropoda—

- Cellana tramoserica* (Chemnitz)*
Patellanax squamifera (Reeve)*
P. perplexa (Pilsbry)
Haliotis ruber (Leach)
Scutus antipodes Montfort
Tugalia parmophoidea Q. & G.

Actiniaria—

- Actinia tenebrosa* Farq.*
 (best developed at higher levels)
Anthopleura (*Oulactis*) *muscosa* (Drayt.)*
Bunodactis (*Phymactis*) *veratra* (Drayt.)*
Anthothoe albocincta (Hutt.)
Phlyctenanthus australis Carlg.

Corallimorphidae—

- Corynactus australis* Hadd. & Duerden

Worms

- Platynereis dumerilii* (Aud. & M. Edwards)
Pseudonereis anomala Grav.
Syllis closterobranchia Schmarda var. *kinbergiana* Augener
Syllis zonata Haswell
Eusyllis (near *kerгуелensis* McIntosh)
Audouinia anchylochaeta (Schmarda)
Lepidonotus melanogrammus Haswell*
L. bowerbankii Baird*

Turbellaria—

- Leptoplana australis* Laidlaw
Diplosolenia Johnstoni Haswell

Bryozoa

- Watersipora cucullata* (Busk)
Cantenicellid sp.
Cryptozoon sp.

Mollusca

- Eligidion audax* Ired.
Sophismalepas (*Lucapinella*) *nigrita* (Sowerby)*
Bankivia (*Cantharidus*) *fasciatus* (Menke)
Bellastraea sirius (Gould) (olim *Astraea tentoriiformis* Jonas)*
Ninella torquata (Gmelin) (olim *Turbo stamineus*)*
Turbo militaris Reeve
Ravitronea (*Cypraea*) *caputserpentis* (Linné)*
Charonia rubicunda (Perry)
Cymatilesta spengleri (Perry)

Gastropoda (*continued*)—

Mayena australasia (Perry)
Monoplex australasiae (Perry)
Nassarius particeps (Hedley)*
Morula (Drupa) marginalba (Blainville)*
Floraconus papilliferus (Sowerby)*
Umbraculum botanicum Hedley*
Flabellina janthina Angas*
F. ornata Angas*
Aeolis (Coryphella) macleayi (Angas)*
Chromodoris (Glossodoris) bennetti
 (Angas)*
Casella atromarginata (Cuvier)*
Ellsiphon scabra (Reeve)*

Pelecypoda—

Mytilus obscurus Dunk.*
Trichomya (Brachydontes) hirsutus
 (Lamarck)*
Codakia rugifera (Reeve)
 (only where there is deep sand)
Arca pistachia Lam.*
Austrolima nimbifer Ired. (olim *Lima*
multicostata Sowerby)
Lasaea australis Lamarck*
Venerupis crenata Lamarck

Crustacea

Decapoda—

Caridea—

Rhynchocinetes rugulosus (Stimpson)
Leander serenus Heller

Naxia tumida (Dana)

Pilumnus rufopunctatus (Stimp.)

Eriphia norfolcensis McCull.

Brachyura—

Plagusia capensis de Haan*
P. glabra Dana*
Pachygrapsus transversus Gibbs
Halicarcinus varius (Dana)

Anomura—

Eupagurus lacertosus Hend.*

Amphipoda—

Isopoda—

Insecta

Clunio pacificus Edw.

Echinodermata

Asteroidea—

Patiriella calcar (Lam.)*
P. gunnii (Gray)*
Allostichaster polyplax (M. & T.)*
Coscinasterias calamaria (Gray)*
Uniophora granifera (Lamarck)
Asterina inopinata Livingstone*

Echinoidea—

Helicidaris tuberculata (Lamarck)
Holopneustes pycnotylus H. L. Clark
 (usually in fronds of the kelp;
 seasonal)
Tripneustes gratilla (Linné) (seasonal)
Phyllacanthus parvispinus Tenn. Woods

Ophiuroidea—

Ophionereis schayeri (M. & T.)*
Ophiocoma pulchra (H. L. Clark)*
Ophiarachnella ramsayi (Bell)*
Placophiothrix spongicola (Stimp.)*
Ophiactis savignyi (M. & T.)*

Crinoidea—

Cenolia trichoptera (Müller)

Holothuroidea—

Stichopus mollis (Hutton)

Chordata

Ascidacea—

Cynthia sp.*
Botrylloides sp.

Enteropneusta—

Balanoglossus australiensis (Hill)

Of the types named above, the following are worthy of a further note. Some of the large gastropods are common members of the littoral-sublittoral zones. Characteristic on very exposed rocks is the "cart-rut" shell, *Dicathais orbita*. It is amazing how this creature stands up to the waves and seems to prefer the surf-beaten headlands. It may be seen laying its eggs in July and August. At the same levels one finds *Haliotis ruber*, but only where there is shelter, and particularly in somewhat sheltered pools or hollows or under ledges where the sea has eaten into the edges of rock platforms. Large specimens may be found on exposed surfaces at very low levels, especially on the sides of crevices, and small ones may be found under stones in rock pools. At the same low levels are very large tritons (*Cymatillesta spengleri* and *Charonia rubicunda*) and the black Fissurelid, *Scutus antipodes*. The tritons may crawl about in exposed places, but *Scutus* likes a deep pool to which the sea has entrance, yet where there is perfect protection, and often still water when the tide is out. The large turban shell, *Ninella torquata*, is also found here. Along the northern part of the coast, north of Nambucca, another large turban shell, *Turbo militaris*, appears. Occasional specimens turn up as far south as Long Reef.

At the upper margin of the littoral-sublittoral fringe and where the *Pyura* zone passes into the *Galeolaria* zone one finds (especially on horizontal platforms) two large chitons, *Poneroplax paeteliana* and *Onithochiton quercinus*, which are to be seen fully exposed on the surface (except in extreme south where *O. quercinus* is missing). Less frequently, some specimens of *Poneroplax* may be found at higher levels than this, but in pools in a wild splash zone. Most of our chiton species hide away under stones, but this does not apply to the two species just named, nor to *Sypharochiton* or *Liolophura* which prefer higher levels. Thus, three easily visible chiton species (for up to date we have not found *Sypharochiton* and *Liolophura* together at one place) occupy well-defined zones on the shore.

One should mention at this point the most striking of all our worms—the giant tube-worm, *Sabellastarte indica*. The finest collection—hundreds of expanded heads looking like a flower bed—was seen in this zone below the *Galeolaria* at Merewether just south of Newcastle, but this worm often occurs in permanent rock pools at high intertidal levels on rock platforms, *provided* the pools are near a splash zone and kept filled with ever changing sea-water.

Sabellastarte indica is the polychaete which Haswell described in error as *Spirographis australiensis*, and up to the present date, this worm, known to all students, has been called by that name, although several authors, writing on collections of polychaetes from other places, have questioned Haswell's diagnosis (Augener 1914-1930; Fauvel 1917). We have been able to obtain very fine specimens of this glorious tube-worm and have seen hundreds *in situ* all along the New South Wales coast. The species has also been examined in the preserved state. There can be no question about its being *Sabellastarte indica*, which has actually been recorded by overseas collectors from several places in the neighbourhood of Sydney.

At about the level of the *Pyura* zone or the lower part of the *Galeolaria* zone (where a horizontal surface exists at this level), one often meets isolated specimens of a polychaete which constructs a thick and very hard, irregular tube of cemented sand. The worm is the species *Idanthyrus pennatus*. At Norah Head a mass of these tube-worms was found at one place. At Nambucca Heads, further north, several masses were found at a level definitely below the *Galeolaria* and thus in the fringe zone. At Angowrie, still further north, however, a very great development of this mass formation occurred. Here, below the *Galeolaria*, almost all the great blocks of rock which form the southern margin of the bay are cemented together by *Idanthyrus* tubes.

As already noted, the characteristic barnacle of the littoral-sublittoral fringe is the very large *Balanus nigrescens*. This species likes plenty of surging water, and is influenced by the presence of breaking sea and spray so that it may occur at higher levels if special conditions combine to favour it. The highest occurrence we have seen was at a spot where a narrow crevice about six inches wide formed a funnel up which the sea rushed at every breaking wave. Obviously *B. nigrescens* will not be found far away from the edge of a rock platform.

It is not our intention in this paper to give lists of *all* the species found in the different zones. Of special note, however, in the littoral-sublittoral fringe are the encrusting sponges and two small anemones which are particularly characteristic. The sponges are, unfortunately, not identifiable with certainty. The commonest one is of a heliotrope colour (probably *Haliclona* sp.), and other common forms include a *Chondrilla*-like species and several irregular encrusting forms of bright colours. A *Tethya*, *Tethya corticata* Lend. (= *T. diplodetma* Schmidt), is also common.

The anemones need special mention. One small orange-coloured species, *Corynactus australis*, the club-shaped tentacles of which end in little whitish knobs, occurs in thousands, with the individuals touching each other and covering the rocks. It is found round about zero tide mark and is rarely uncovered by the sea. It prefers some shelter, however, and is usually seen under ledges, on the sides of some gutter, rock pool open to the sea, or other big crevice formed by the action of the waves. This is a highly characteristic species of the littoral-sublittoral fringe and should attract the attention of anyone making a thorough examination of the shores at extreme low-water. Up to date it has not been recorded as playing a distinctive part on our shores. The common anemones, *Actinia tenebrosa*, *Anthopleura muscosa*, and *Bunodactis veratra*, may all be found in the littoral-sublittoral fringe, but they are more frequent in other zones.

(b) Mid- and Upper-Littoral

III. The *Galeolaria* Zone

It must be emphasised again that in speaking of a *Galeolaria* zone we are referring to the zone marked by *Galeolaria caespitosa* in a closely packed, encrusting condition, just above the *Pyura* zone. As is usual with porous material

the encrustation provides a home for other organisms, and as we have already noted, the whole mass becomes a fascinating ecological formation in which certain arthropods, molluscs, and worms live in a balanced community.

Actually the level of the *Galeolaria* zone is, for every-day collecting, the most luxuriant of all, but this applies only where a horizontal rock platform surface occurs at this level. The reason, of course, is that the still richer lower levels require the rarer coincidence of the lowest tides and favourable weather for their examination. Naturally, where the *Galeolaria* encrustation is presented on a vertical or near vertical face, there is a much more restricted fauna consisting almost solely of the species enclosed within it, together with a few chitons and gastropods.

The *Galeolaria* association exemplifies many of the most interesting problems of intertidal ecology which are unsolved. There is first the problem of the factors causing its sharp limitation in the encrusting condition. A considerable amount of observation around the coast is necessary to appreciate the conditions involved and the factors which favour a full expression of its growth. *Galeolaria* may be found living over a wide series of shore zones. Isolated individuals may be seen many feet above the level of what we have called the *Galeolaria* zone and many yards away from the seaward margin of a rock platform, but under these conditions they are always distinctly separate and scattered and are in places which remain wet with sea-water when the tide recedes. The most surprising occurrence of this kind was a single, isolated specimen alive in a very small rock pool high up (approximately 12 ft. above its usual level) on the shore at Harbord. The pool was, however, in a splash zone where it might be kept reasonably saline, despite rain.

We have already stressed the fact that as a rule the *Galeolaria* encrustation forms a band, with the upper limit about mid-tide level and the lower limit abutting against the *Pyura*. This condition is not only widespread, but also may be seen at places as different as estuary mouths and open ocean rock faces. Yet there are apparently at least two other variations in type of occurrence. One of these, seen clearly at the extreme tip of Long Reef, has been described on page 197. It is a variation in which the upper limit of the *Galeolaria* is considerably raised and the growths are vigorous. On the other hand, there are places on the extreme exposed coast where heavy wave action seems too much for *Galeolaria* and a growth of algae such as *Pterocladia* and corallines takes its place. Finally, there are a few localities, sometimes extremely sheltered, and often where the rocky coast abuts on sand beaches, where *Galeolaria* is again repressed and one either finds the rock surfaces invaded by the small barnacle, *Chamaesipho* (usually so abundant in the zone immediately above the *Galeolaria*), or occupied by a close growth of the scrubby *Corallina*, *Padina*, *Gymnosorus*, and stunted *Sargassum* (see Fig. 3, Column IV).

Many years ago Hedley referred to the *Galeolaria* as occurring both on surf-swept headlands and on wharf piles in sheltered water, but said it was intolerant of sand or mud. He also recognized that in places its crust might be six to eight inches thick and specially mentions Wyargine Point at the entrance of Middle Harbour for this condition.

We have seen far greater developments than this at Grant's Head, but nowhere on the coast of New South Wales does *Galeolaria* flourish so well as at Merewether, just south of Newcastle.

Investigation of the Merewether formation with comparisons and close observations at other places suggests the following conclusions:

Galeolaria caespitosa likes continuous moisture and, in particular, requires to be submerged for a considerable period each day. It thrives best in ocean water but not where heaviest wave action is prevalent. It does not mind broken surf and in very exposed places an outer rampart of fallen and half-submerged rocks is quite enough to provide excellent conditions for its growth on the lee or semi-lee sides. These are the conditions at Merewether where the growth is thickest just away from the most exposed margins of the rock platform.

The fine growths at Merewether and other places are not far from a sandy part of the shore. The proximity of sand is thus not an obstacle to its growth.

Even the saline calm water of Sydney Harbour in Rose Bay and below the zoological gardens is still favourable to considerable growths, but a short distance above the Spit Bridge in Middle Harbour it is very poorly represented, whilst the long seven mile arm of the Hawkesbury estuary—Pittwater—seems almost poisonous to it, compared with the certainly polluted water of Sydney Harbour. The water of Pittwater is probably less saline in wet weather, but the more obvious difference is that the water of the Hawkesbury entrance and Pittwater contains much fine mud suspended in it.

The exceptional extent of its growth at Merewether is difficult to explain and we have no clear proof available of any causal factors. Merewether ocean beach is, however, near Newcastle, and a prominent stormwater (street drainage) channel opens on the shore nearby. Wyargine Point makes an interesting comparison for it is directly opposite the ocean entrance to Sydney Harbour and at one side of Balmoral Beach. It receives surf, yet is moderately well sheltered, and it is also in proximity to some drainage water. On the whole, however, we are intrigued at the subtle reaction to some chemical or physical feature in the environment.

One might possibly expect sharp tidal limits where *Galeolaria* is found growing on a wharf pile or on rocks in a somewhat sheltered harbour situation. One can also conceive of a certain degree of exposure to the air being fatal, so that the upper limit could be directly related to tidal levels. But the problem is made much more puzzling by the sharp limit seen even where the sea surges up and down almost continually over the rocks on the ocean coast (see Plate 6). Varying degrees of exposure do not affect the levels very much. And this applies similarly to the sharp "cut-offs" of the large brown seaweeds and to *Pyura*.

The only explanation we can offer at present is the following: We suggest that *Galeolaria caespitosa* is, in general, adapted to a fairly prolonged period of submergence under water. Let us ignore the average days of surf and swell with spray, and consider only the exceptional days with no swell. On these occasions the limiting factor for the upper "cut-off" could very well be tide level. In support of the contention that it may be these exceptional conditions which are limiting factors we might point to the fact that on one occasion only during the year 1945-6 there was a particular kind of blinding white band along part of the very exposed coast of New South Wales between lowest tide levels and mean-tide level. Examination showed that it was due to dead seaweeds, bleached white in the sun. These were chiefly corallines and *Ulva* which normally live satisfactorily in this region because of spray and surf. At this date, however, a series of low spring tides had coincided with an extremely calm ocean and cloudless days, and the result was fatal. (One might note here that it is not the summer temperature which limits the southward extension of coral reefs, but the winter temperature.) Incidentally, the upper limit of thick *Galeolaria* encrustment coincides almost with the lowest level of high water at neap tides.

ANIMALS CLOSELY ASSOCIATED WITH GALEOLARIA ENCRUSTATIONS

Species marked with an asterisk are also found at other levels.

Coelenterata

Actiniaria—

Actinia tenebrosa Farq.*

Worms

Gephyrea—

Physcosoma japonica (Grube)*

Polychaeta—

Phyllodoce sp.

Nereis sp.

Turbellaria—

A Leptoplanid worm (sp. not yet determined; very common everywhere *Galeolaria* examined)

Nemertinea—

Sp. undetermined.

Arthropoda

Arachnida—

Desis crosslandi Pocock

Pycnogonids—

Crustacea—

Ibla quadrivalvis Cuvier*

Mollusca

Loricata—

Meturoplax retrojecta Pilsbry*

Pelecypoda—

Lasaea australis Lamarck*

Brachydontes hirsutus (Lamarck)*

Mytilus obscurus Dunk.*

Gastropoda—

Onchidium patelloides Q. & G.*

It should be stressed that in the list above only those animals found very closely associated with the thick encrustations, often only discovered by breaking

off clumps of tubes, have been given. Migratory species such as gastropod molluscs, crabs, etc., which may be found at one place or occasion and not at another, are all noted in the next list.

Of the animals listed above, a special note is desirable in so far as the following species are concerned: *Desis crosslandi*, *Onchidium patelloides*, *Lasaea australis*, and *Ibla quadrivalvis*. The spider, *Desis crosslandi*, has only been found by us in *Galeolaria* encrustations. The other three species, although occurring elsewhere on the shore, are particularly at home in the *Galeolaria* and are abundant at least from Merewether to Ulladulla.

The Marine Spider, *Desis crosslandi* Pocock

This spider occurs with a web nest between the intertwined *Galeolaria* tubes and so is almost completely buried in the encrustation. The genus is surely outstanding, especially in view of the rarity of members of the group Insecta and of spiders in the sea. Several species of *Desis* are known (Pocock 1902), and despite the fact that they have been found in widely separated parts of the world, the shore zone in which they have been taken presents considerable similarity in tide level. Thus *Desis martensi* Koch from the Java Seas occurs in coral or reefs at places "only temporarily uncovered by the sea." One of the first systematists to describe a specimen doubted the possibility of spiders living such a submarine life, and thought they had been floated in an accidental manner from the land. But to get at these spiders the coral has to be broken up, and the same thing applies exactly to *Desis crosslandi* from the *Galeolaria*. Strangely enough, this species is recorded from the Barrier Reef and also from Zanzibar. Another species, *Desis marinus*, has been recorded for Port Jackson and this also occurs on the shores of New Zealand. The Port Jackson specimens are stated by Whitelegge to be "a very common species of spider found under stones at low-water mark." Of the New Zealand specimens it is said that "all the spiders of this kind which we have found have had nests in holes" (*Lithodomus* holes in the rock) "and always under water at all times of the tide" (Robson 1877).

No reference has been made before to the occurrence of *Desis* in *Galeolaria* encrustations and yet we have never found the spiders elsewhere. This is the first record for *Desis crosslandi* from the coast of New South Wales.

Still another species of *Desis*, *D. Kenyonae*, has been collected and described from east Australian shores—from Westernport in Victoria. Mrs. Kenyon, the discoverer of the Victorian specimens, says she found a specimen under a sea-worm shell which seemed attached to the rock by web and it appears to have been partly submerged under a stone. *D. crosslandi* is reported from the Barrier Reef merely as occurring in holes and crevices of the under-side of stones *which are completely submerged at high water*.

It will be noted, therefore, that here we have a spider living in an encrustation at a fairly low level on the open ocean coasts. It is difficult to see how it could ever reach the surface when submerged and it is in a position where often

the surf beats on its borrowed worm-tube bastions. It would appear that the whole manner of its behaviour is still a problem of a most interesting kind. Whatever may be said about the occurrence in holes under stones it is particularly worthy of note that a South African species, *D. tubicola*, is, like ours, actually found amidst masses of worm tubes—in this case, sandy worm tubes. The description of its occurrence is worth recording in full because what is said applies so closely to our local species. The reference is from Pocock's paper (1902).

"Mr. Nendick Abraham's account of the habits of this spider is reprinted from the 'Bulletin of the Liverpool Museum.' After describing his first discovery of the animal in the tube-masses of *Tubicola*, the writer proceeds: 'This formation (the *Tubicola*-masses) is invariably covered by the sea at high tide, and much of it even at low tide . . . Sometimes I have found five or six spiders in one piece of material weighing five or six pounds. Now, what is curious is that these spiders cannot swim or dive, and when placed on the surface of the water appear to be quite helpless, or nearly so . . . I eventually succeeded in securing several nearly perfect examples (of their dwellings). I then saw that the spider does not, as a rule, make its home in the empty tubes of the worms, but . . . in the spaces left between the tubes. The dwelling consists of a delicate silken chamber with the opening seaward. It is so frail and delicate that the least rough handling destroys it. Yet in this frail home of silk, hidden away in some little space in the masses of tubes built by marine worms, these spiders live and thrive, . . . the waves breaking over them all day long . . . I have watched the tubes when the tide was low in the hope of seeing a spider crawling or running about, but I have never yet seen one. They live out of sight deep down amongst the worm tubes. How they catch their food, what their food is, and how they keep the sea from drowning them, are questions I have not yet demonstrated, though I have tried again and again to keep them in my marine aquaria. Shortly after introducing one, I have often found it floating helplessly on the water, apparently half dead, and I have had it lifted out of the water and placed on the rockwork, when it soon became active and ran about very quickly, when it appeared to be just like an ordinary spider.'"

Incidentally, when species of *Desis* were first discovered, stress was laid on the fact that they were known from South Africa and Australia with a gap in between. The original finding of *D. crosslandi* at Zanzibar was noteworthy in that it helped to bridge the gap. We now record the same species from the south-east coast of Australia.

Ibla quadriavis Cuvier

Ibla is an Indo-Pacific Cirripede genus of the family Scalpellidae, a group of the stalked barnacles which is found attached to rocks or to animals in the littoral. It is a small form only about 20-30 mm. in height including the peduncle. The genus is a small one with few species but it has been of great interest anatomically and physiologically since Darwin wrote of it. The valves are only four in

number and horny, the peduncle is clothed with horny spines which look like coarse hair. The particular interest of the genus, however, is the presence of separate sexes, the males being very small, living inside the mantle cavity of a female or hermaphrodite form practically as parasites. The condition was described by Darwin who actually dissected the tiny complementary males only about one-sixth of an inch long. His specimens came from Kangaroo Island, South Australia, and the description is testimony to his powers of observation. Later the structure of the parasitic males of our species was described in detail by Gruvel in 1904. The *Galeolaria* association provides an almost certain place of finding specimens of *Ibla* in our intertidal zone, but *Ibla* also occurs attached to the lower sides of rocks.

Onchidium patelloides Q. & G.

The discovery of this little marine pulmonate between *Galeolaria* tubes was another very striking incident of this research. The entire group of the Onchidia is particularly interesting and its occurrence had aroused our especial attention in ecological researches on New South Wales beaches before discovering the habits of the species now in question. There are several Australian species, and their common habitat is the shore of estuaries—particularly the muddy shore of mangrove swamps. During these investigations we found that the species, *O. patelloides*, favoured the open ocean coasts. One could not always count on finding it, but on occasions it was quite common on the rock platform at Long Reef. It was next discovered by one of us on the southern coast of Victoria, near Lorne, and so the species has a wide range on the south-east coast of Australia. It came quite as a surprise, however, to find that an *Onchidium* was a regular and common inhabitant of the *Galeolaria* encrustation. The specimens occur amongst the tubes and the flattened shape is evidently very suitable for the home it has found. Most Onchidia seem to feed when the tide recedes and retire to holes when the ground is flooded. Possibly *O. patelloides* does the same, but it is doubtful if it wanders far away from the *Galeolaria*, and it is not usually seen unless a piece is broken off and carefully examined.

Lasaea australis Lamarck

This member of the community is a small bivalve and it may occur in hundreds between the *Galeolaria* tubes. The species is very well known (perhaps best under the generic name of *Kellia* Turton 1822, although *Lasaea australis* was the original name of Lamarck). It occurs all along the south coast of Australia as well as up the western and eastern coasts and it has a habit of fixing itself in crevices and between barnacles. We find it particularly common amidst the corallines of the littoral-sublittoral fringe. The *Galeolaria* encrustation appears to be a second highly favoured place. The shell is only about 6 mm. in length. The genus is noteworthy because of its incubatory habits, the developing eggs being retained in the interlamellar gill spaces until the young are far advanced.

OTHER SPECIES OF THE GALEOLARIA ZONE

(Additional to those already listed in the *Galeolaria* encrustations)

As already stated the rock platform pools and crevices at the level of the *Galeolaria* zone are probably the most favourable collecting grounds of all. The following list indicates other animal species which are common at this level. Some of these may be seen on the rock surfaces. Most occur under stones. Actually, it is rare to find a suitable stone on a rock platform which is not resting in a depression with a little water. In other words, an upturned stone not only reveals a fauna which seeks shade and is definitely negatively phototropic but one which might be correlated also with the necessity for some submergence in water between tides. This must be kept in mind since it complicates matters. The list, however, refers more particularly to animals which are only in very small and shallow collections of water under stones and not to deep rock pools unless specially stated.

Species marked with an asterisk are found at other levels.

Coelenterata

Actiniaria—

Anthopleura muscosa (Drayt.)*
Bunodactis veratra (Drayt.)*

Phlyctenactis tuberculosa (Q. & G.)*
Phlyctenanthus australis Carlg.*

Worms

Polychaeta—

Salmacina australis Haswell*
Serpula sp.
Spirorbis sp.*
Sabellastarte indica Sav.*
Galeolaria hystrix Mörch*
Diopatra dentata Kinberg*
Eunice aphroditois (Pallas)*
E. antennata (Sav.)
E. siciliensis Grube
Syllis variegata Grube
Nereis pelagica Linn. loc. var.
Lepidonotus melanogrammus Haswell*
L. bowerbankii Baird*

L. argus (Qtrfgs.)
Eurythoe complanata (Pallas)
Idanthyrsus pennatus (Peters)*

Turbellaria—

Leptoplana australis Laidlaw*
Diplosolenia johnstoni Haswell
Thysanozoon sp.
Triplocelis typica Hasw.

Nemertinea—

Gorgonorhynchus repens Dakin
 Several unknown spp. are common

Mollusca

Loricata—†

Ischnoradsia australis (Sowerby)*
Haploplax smaragdina (Angas)*
H. lentiginosa (Sowerby)
Ischnochiton elongatus (Reeve)
I. versicolor (Sowerby)
Callistelasma antiqua (Reeve)*
 (particularly where there is mud)

Rhyssoplax jugosa (Gould)*
Cryptoplax mystica Ired. & Hull*
Poneroplax paeteliana (Thiele)*
Sypharochiton septentriones (Ashby)*
Liolophura gaimardi (Blainville)*
Onithochiton quercinus (Gould)*

† With the exception of the last four species all these chitons are found on the under-sides of stones.

Mollusca (*continued*)

Gastropoda—

Cellana tramoserica (Chemnitz)*
Patellanax squamifera (Reeve)*
Scutus antipodes Montfort*
Sophismalepas (*Lucapinella*) *nigrita*
 (Sowerby)*
Montfortula conoidea (Reeve)*
Austrocochlea obtusa (Dillwyn)*
A. concamerata (Wood)*
Stomatella imbricata Lam.*
Gena impertusa Burrows*
Bellastraea sirius (Gould)*
Subnina undulatus (Mart.)*
Ninella torquata (Gmelin)*
Melanerita melanotragus (Smith)*
Melaraphe unifasciata (Gray)*
Bembicium melanostoma (Gmelin)*
Bittium lacertinum Gould*
Ravitriona (*Cypraea*) *caputserpentis*
 (Linn.)*
Ellatrivia merces Ired.*
Vicimitra contermina Ired.
Nassarius particeps (Hedley)*
Dicathais orbita (Gmelin)
Bedeia (*Xymene*) *hanleyi* (Angas)*
Morula marginalba (Blainville)*
Agnewia tritoniformis (Blainville)
Floraconus papilliferus (Sowerby)*

Bullinula lineata Brazier (seasonal)
Dolabrifera brazieri Sowerby
Tethys angasi (Sowerby)
T. norfolkensis (Sowerby)*
Umbraculum botanicum Hedley
Pleurobranchus punctatus Q. & G.*
Flabellina janthina Angas*
F. ornata Angas*
Aeolis (*Coryphella*) *macleayi* (Angas)*
Chromodoris (*Glossodoris*) *bennetti*
 (Angas)*
Casella atromarginata (Cuvier)*
Dendrodoris davisii Allan
Ellisiphon scabra (Reeve)*
Talisiphon virgulata (Hedley)*
Onchidium patelloides Q. & G.

Pelecypoda—

Arca pistachia Lam.*
Anomia descripta Ired.*
Marikellia solida (Angas)
Venerupis crenata Lamarck*

Cephalopoda—

Octopus cyaneus Gray*
O. maculosus Hoyle*

Crustacea

Decapoda—

Caridea—

Crangon strenuus (Dana)
Alope australis (Baker)
Leander serenus Heller

Brachyura—

Naxia tumida (Dana)*
Pilumnus rufopunctatus Stimp.*
Ozius truncatus M. Edw.*
Leptograpsus variegatus Fabr.*
Pachygrapsus transversus Gibbs*
Plagusia capensis de Haan*
P. glabra Dana*

Anomura—

Eupagurus sinuatus Stimp.
E. lacertosus Hend.*
Paguristes squamosus McCull.
Clibanarius taeniatus M. Edw.

Amphipoda—

Isopoda—

Cirripedia—

Tetraclita purpurascens (Wood)*
Chamaesipho columna (Spengler)*
Tetraclita rosea Darwin*
Catophragmus polymerus Darwin
Balanus imperator Darwin*
Elminius simplex Darwin*

Echinodermata

Asteroidea—

Patiriella exigua (Lamarck)**P. calcar* (Lamarck)**P. gunnii* (Gray)**Asterina inopinata* Livingstone**Allostichaster polyplax* (Müll & Tros.)**Coscinasterias calamaria* (Gray)**Ophiarachnella ramsayi* (Bell)**Placophiothrix spongicola* (Stimp.)*

Echinoidea—

Heliocidarid erythrogramma (Val.)**Tripneustes gratilla* (Linn.)*

Ophiuroidea—

Ophiocoma pulchra H.L.C.**Ophionereis schayeri* (Müll. & Tros.)*

Holothuroidea—

Leptosynapta dolabrifera (Stimp.)**Pentacta australis* (Ludwig.)*

Chordata

Ascidiacea—

Cynthia sp.**Sidneioides tamaramae* Kest.

Other compound ascidians

Enteropneusta—

Balanoglossus australiensis (Hill)

IV. The Barnacle Zone

The succession of the indicator barnacles and the factors which modify the succession in rough, exposed, or calm and sheltered localities have already been described.

In this section it is proposed to consider some aspects of the general ecology of the whole strip of intertidal shore above the *Galeolaria* zone. Referred as near as can be to tide levels, this means approximately the upper half of the intertidal belt of the shore, and its lower limit may well be the lowest level touched at high-water of neap tides.

The greater part of the area of most of the New South Wales rock platforms falls into this zone, and consequently it is an area of shore which is familiar to the general public. It is characterized, except north of Ballina, by the presence of the small barnacle, *Chamaesipho columna*. The upper limit may be indicated by a change from this to *Chthamalus*, but the growth of *Chthamalus* is restricted and varies considerably even within its narrow limits. In many places close encrustations of *Chthamalus* are rare, in a few localities one can find large areas closely covered. An excellent example of this was found at Yamba, and it may also be seen at other places on the coast.

The surfaces of rock platforms lying at the lower levels of the *Chamaesipho* zone often present rich growths of *Hormosira Banksii*, which may also invade the area occupied by the *Galeolaria* encrustations. This is a brown alga which is a

characteristic species of the South-West Pacific and only seems known from New Zealand and Australia. It is one of the most striking algae of the New South Wales intertidal zones, and in places may be thick enough to form what has been called a *Hormosiretum*. We have never found growths as rich, however, as the coverings of *Fucus* on British coasts. *Hormosira* evidently likes to be moist and yet to be exposed for a short time either to air or the unfiltered rays of the sun. Its habitat clearly reflects these requirements, for where a platform surface is somewhat convex and tends to dry up soon after the tide recedes, it will be found only as thick fringes round shore pools. Here, however, it still reveals its character as a mid-littoral form because it fails to grow in permanent shore pools below a depth of six or more inches. It just forms a curious thick fringe round the margin—its “beads” making contact with the air whilst the tide is out (see Plate 2).

Also, in places where water tends to collect, in juxtaposition with the *Hormosira*, are close mats of *Corallina*. Now *Corallina* is characteristic of the kelp zone and the littoral-sublittoral fringe. It is therefore of particular interest to note that this *Corallina* which is at a higher level is very different in appearance. It is dull and dirty looking in colour and forms low, scrubby growths which look like the surface of an old and much worn nail-brush. It feels coarse and brittle and particles break off. One might have expected this stunted *Corallina* to be a different species from any in the lower shore zones, especially as it is usually found quite separated from those zones. Up to date we have been given to understand that it is the same species at both levels, and in places a continuous series does seem to exist between that of the high zone and that of the kelp beds.

On well-exposed margins of platforms in this zone are moderate growths of *Ulva*. Away from this, and near the land side on the highest rock surfaces of the intertidal zone, the whole surface may be covered with a dazzlingly brilliant green growth of *Enteromorpha*. This is especially the case where an oozing or small drainage of fresh-water seeps from the land. Other surface algae of the higher parts of the platforms are *Bangia* and *Ectocarpus*. There are, it should be noted, very rich and varied growths of algae in the deeper and more extensive rock pools of the lowest parts of the zone.

The characteristic surface-loving chiton, *Sypharochiton septentriones*, marks the *Chamaesipho* zone but only along the central stretch of the coast. Specimens are practically always found in little shallow depressions of the rock surface and so very frequently have no shade from the vertical sun, although presumably they obtain moisture and a somewhat sheltered spot in this way. Reference has already been made to the interesting geographical distribution of this species.

Glancing over the often broad area of level rock, much of which is covered by *Chamaesipho*, one notices that up to a certain distance from the seaward margin considerable areas of the flat rock surface (wherever a slight concavity results in shallow collections of water remaining between tides) bear regularly

scattered individuals of *Galeolaria*. And, also distributed over the whole area are numerous limpets, of which *Cellana tramoserica* is most conspicuous, together with the pulmonate *Ellsiphon scabra*, which simulates a limpet in appearance.

The other, more obvious and exposed animals are six or seven species of gastropods, very large numbers of which occur browsing on the algal scum of the rock surface. There is a very clear succession in the distribution of these air-loving species as one passes from the seaward margin towards the land. Lowest are *Austrocochlea concamerata* and *A. obtusa*, along with the carnivorous *Morula marginalba*. Then come *Melanerita melanotragus*, *Bembicium melanostoma*, and uppermost, the little blue *Melaraphe unifasciata*. These last two are Littorinids.

As the sea-water recedes from the surfaces of the rock platforms it is interesting to watch the species which are relatively quick movers. They gradually move to the wetter places and to shade. Most characteristic, however, is the tendency of the species on higher areas to go into a "huddle." One meets collections of *Melanerita* and *Bembicium* each consisting of dozens of individuals. Most striking, however, in this respect is *Melaraphe* which is usually present in thousands. It collects or huddles in groups wherever there is a corner, a hollow, or even the slightest ridge on the rock surface.

In some places where there are collections of small stones and boulders, one gastropod species may occur in pockets beneath them literally in hundreds. This is *Hinea brasiliana*. Possibly it is one of the most common univalves of the shore, yet along many rock platforms it may never appear conspicuously.

Scattered about at this upper margin of the littoral are specimens of the rock oyster, *Saxostrea commercialis*, but the specimens are small compared with those of the estuaries. The oysters mark a high zone of the intertidal shore and their zoning is fairly sharp, a character which is brought out very conspicuously in the estuaries. The photograph (Plate 9) shows the oyster's relationship to *Hormosira* in this respect.

A very different habit is that of the other common barnacle of the rocky shores—*Tetraclita purpurascens*. This species seems entirely unaffected by tide levels. Its needs are moisture and shade, and if a corner under an overhanging rock or crack provides the favourable conditions, one may meet collections of *T. purpurascens* at practically any level on the shore from the *Pyura* zone to the supralittoral.

In cracks and channels running across the level parts of rock platforms where sea-water remains, one finds the three anemones, *Actinia tenebrosa*, *Anthopleura muscosa*, and *Bunodactis veratra*. They are actually found in all zone levels. The red species, *Actinia tenebrosa*, is, however, most at home in the highest intertidal zones and one frequently finds collections of individuals left far away above low-tide level on the sides of a platform boulder. Here, in the retracted state, they wait for a return of the tide.

One other species of the highest parts of the intertidal zone may be specially mentioned as one of the types which may be seen fully exposed. This is the little sea-star, *Patiriella exigua*, which thus differs entirely from its two relatives, *P. calcar* and *P. gunnii*, which prefer rock pools of the lower zones.

MOST COMMON SPECIES OF THE ROCK SURFACES IN THE BARNACLE ZONE

Coelenterata

Actiniaria—

Actinia tenebrosa Farq.

On surface rocks

Bunodactis veratra (Drayt.)

Anthopleura muscosa (Drayt.)

On surface, but usually in cracks with a little residual water

Worms

Polychaeta—

Diopatra dentata Kinberg

Usually exposed, but in cracks with water and sand

Galeolaria caespitosa Lam.

Isolated tubes in moist places

Idanthyrsus pennatus (Peters)

Few isolated specimens in wet places

Mollusca

Loricata—

Sypharochiton septentriones (Ashby)

Subnirina undulatus (Mart.)

In pools

Gastropoda—

Notoacmaea petterdi (Tenn. Woods)

Melaraphe unifasciata (Gray)

Cellana tramoserica (Chemnitz)

Montfortula conoidea (Reeve)

Melanerita melanotragus (Smith)

Austrocochlea obtusa (Dillwyn)

A. concamerata (Wood)

Bembicium melanostoma (Gmelin)

Bellastrea sirius (Gould)

In shallow pools

Dolabrifera brazieri Sowerby

Over a wide area of surface, and in pools and under stones, but seasonal and wandering over platform in breeding season (October-March)

Ellsiphon scabra (Reeve)

Talisiphon virgulata (Hedley)

Pelecypoda—

Saxostrea commercialis Ired. & Rough.

Crustacea

Chamaesipho columna (Spengler)

Except in the far north

Tetracrita rosea Darwin

Isolated specimens in wet places. In splash and very wet places it may be very thick (except in the far south)

Tetracrita purpurascens (Wood)

At all levels in shade and moist cracks

Chthamalus antennatus Darwin

Only at highest levels

Echinodermata

Patiriella exigua (Lamarck)

In any shallow pools or small cracks

MOST COMMON SPECIES OF THE BARNACLE ZONE WHICH ARE USUALLY HIDDEN UNDER STONES

Since the whole vertical width of this zone may be only a matter of inches, one may find the same organisms scattered over quite a wide area of platform if the level happens to fall in this zone. Actually this is frequently the case. Excellent examples are the major part of Long Reef platform, at least half of the Newport platform, and the parts of the Norah Head platform which are awash at high tide.

Polychaeta— <i>Spirorbis</i> sp.	Worms Turbellaria— <i>Leptoplana</i> sp.
Loricata— <i>Haploplax lentiginosa</i> (Sowerby) <i>Ischnochiton elongatus</i> (Sowerby) <i>I. versicolor</i> (Sowerby) <i>Ischnoradsia australis</i> (Sowerby)	Mollusca Gastropoda— <i>Gena impertusa</i> Burrows <i>Bedevea hanleyi</i> (Angas) <i>Scutus antipodes</i> Montfort Small specimens and under stones <i>Hinea brasiliana</i> Lam.
Decapoda— <i>Leander serenus</i> Heller <i>Naxia tumida</i> (Dana) <i>Ozius truncatus</i> M. Edw.	Crustacea <i>Leptograpsus variegatus</i> Fabr. <i>Pachygrapsus transversus</i> Gibbes
Asteroidea— <i>Patiriella exigua</i> (Lamarck) Often exposed but always in pools <i>P. calcar</i> (Lamarck) At lower levels <i>Coscinasterias calamaria</i> (Gray)	Echinodermata Ophiuroidea— <i>Ophioneis schayeri</i> (Müll. & Tros.) <i>Placophiothrix spongicola</i> (Stimp.) Holothuroidea— <i>Leptosynapta dolabrifera</i> (Stimp.)

(c) *The Supralittoral*V. *Littorinid Zone*

As in other parts of the world, the highest parts of the shore, those above all ordinary tide levels, are characterized by the presence of Littorinids. There are two species of these, *Melaraphe unifasciata* and *Nodilittorina tuberculata*. They may be found on dry rock on which a blazing summer sun pours its rays. *Nodilittorina* is the perfect type of this zone and the highest marine mollusc of the shore. As already noted it has been seen 40 ft. above tide levels and marked specimens have not moved down from this level through periods of several months. At places where there is average shelter the supralittoral with its characteristic mollusc, *Nodilittorina*, will be found at a level of about 6 ft. (approximately just above high-water spring tides) and so one may see *Nodilittorina* on the tops of the very large isolated boulders which are frequent on some platforms. Where there is much splash the lower limit of the *Nodilittorina* will be definitely much higher. *Melaraphe unifasciata*, which is not, however, found so high as

Nodilittorina, is, as already described, a common individual in lower zones, more particularly the higher intertidal levels. Thus, of our three common Littorinids, *Bembicium*, *Melaraphe*, and *Nodilittorina*, the first is restricted to the upper parts of the barnacle zone (upper intertidal); *Melaraphe* occurs at the same levels, but also higher (and in the juvenile condition lower); and *Nodilittorina* is best adapted to the conditions of dry land. Other common species of these high zones of the shore are given in the attached list.

COMMON ANIMALS OF THE SUPRALITTORAL

Species marked with an asterisk also occur in lower zones.

Mollusca

Gastropoda—

Nodilittorina tuberculata (Menke)

Melaraphe unifasciata (Gray)*

Melanerita melanotragus (Smith)*

Notoacmaea petterdi (Tenn. Woods)*

Arthropoda

Crustacea

Amphipoda—

Talorchestia novae-hollandiae Stebbing
and other species

Decapoda—

Cyclograpsus audouinii (M. Edw.)

Leptograpsus variegatus (Fabr.)*

Insecta

Rock pools well up in the supralittoral containing water which ranges from fresh rain water to water of a high salinity due to wave splash and subsequent evaporation are often crowded with the larvae of the mosquito *Aedes* (*Pseudoskusea*) *concolor* Taylor.

It should be pointed out that many zoology students, in their urgency to reach the lower zones, often fail to realize the high level which may be attained by some of the species of lower zones which habitually collect under stones in shallow pools.

The most striking zonation is naturally presented by those forms which are fixed or semi-fixed and must face the extreme conditions of the shore. Animals which favour and can find even shallow pools and sheltering stones may obtain satisfactory conditions (at least for a time) at almost any level.

Thus, careful collections made over long periods may result in the discovery of many species having cryptic habits at many very different levels on the shore.

7. CONCLUSIONS

We have shown how, along the entire rocky coast of New South Wales (and the limits can almost certainly be extended southwards into Victoria and north across the Queensland border) there is a fundamental basic zonation of typical indicator animal species. This zonation is described in the text.

Of the common animal species of the shore zones only a few do not extend throughout this stretch of coast. This is a strong confirmation of the conclusions reached by the systematists working on individual groups (chiefly with collections of echinoderms (Lyman Clark 1946) and molluscs (Hedley 1904; Iredale and others)). On their work, the Peronian province of the Australian coast extends from about 26°S. (Queensland coast) to eastern Victoria.

This basic zonation has been described first for an average condition of exposure. In places where conditions of extreme exposure prevail or where specially sheltered rock margins occur, the basic zonation is slightly modified, but any change is usually an easily recognized variation in which rough surf and splash extend some of the zones vertically, or shelter may favour or repress certain species. The typical zonation is not obscured in such places.

Stephenson (1936-1947), in his excellent reports on the zonation of the South African coast, states that a comparison with Ricketts and Calvin's semi-popular work (1939) on the intertidal shores of California reveals "much that is reminiscent of South Africa." We find much that is reminiscent not only of South Africa and California, but of other temperate coasts. We have, in fact, noted as an exceedingly interesting feature of our zonation studies, the repetition of seashore pictures of other and distant world shores. One might suggest that such broad and interesting resemblances have often been lost in the accumulation of specific names and studies which draw attention to the geographical range of particular species and emphasize the discontinuities of distribution.

For example, we may take the presence of Littorinids on the highest zones of the shore. Actually, the relation of Littorinids to upper zones of rocky coasts is one of these general features which has attracted notice already. The linking of Littorinid species with special zones has received special attention in England, but the presence of Littorinids high in the supralittoral has been noted from Japan, America, and Africa, as well as Sydney. At the other extreme level of the intertidal area we find the kelp, here as elsewhere, marking the lowest levels with the genera *Ecklonia* and *Phyllospora*-species which play the part of *Ecklonia* and *Laminaria*, etc., on South African and other coasts. We have not seen in the northern hemisphere anything like our beds of *Pyura*, but these closely resemble those of South Africa and New Zealand and probably South America, even though the species are recorded as different.

On our exposed rocky shores at low-tide levels we find rock honeycombed with hemispherical hollows containing the purple sea urchin, *Heliocidaris erythrogramma*. We can read an almost identical description of the purple *Strongylocentrotus purpuratus* on the Californian coast. But the same story has been told about rock-boring sea urchins in the surf zone of Europe, and the genera are not so distant from each other; in fact, the names *Strongylocentrotus*, *Heliocidaris*, *Echinus*, and *Echinometra* have often been interchanged in taxonomic history.

Species of the mollusc, *Thais*, are listed for South Africa and the Pacific coast of America at the same low-tide levels as those frequented by *Dicathais* here.



Fig. 1



Fig. 2

DAKIN, BENNETT, and POPE.—A STUDY OF CERTAIN ASPECTS OF THE ECOLOGY OF THE
INTERTIDAL ZONE OF THE NEW SOUTH WALES COAST



Fig. 1



Fig. 2

DAKIN, BENNETT, and POPE.—A STUDY OF CERTAIN ASPECTS OF THE ECOLOGY OF THE
INTERTIDAL ZONE OF THE NEW SOUTH WALES COAST



Fig. 1



Fig. 2



Fig. 1



Fig. 2

DAKIN, BENNETT, and POPE.—A STUDY OF CERTAIN ASPECTS OF THE ECOLOGY OF THE
INTERTIDAL ZONE OF THE NEW SOUTH WALES COAST



Fig. 1



Fig. 2



Fig. 1



Fig. 2

DAKIN, BENNETT, and POPE.—A STUDY OF CERTAIN ASPECTS OF THE ECOLOGY OF THE
INTERTIDAL ZONE OF THE NEW SOUTH WALES COAST



Fig. 1



Fig. 2

DAKIN, BENNETT, and POPE.—A STUDY OF CERTAIN ASPECTS OF THE ECOLOGY OF THE
INTERTIDAL ZONE OF THE NEW SOUTH WALES COAST



Fig. 1

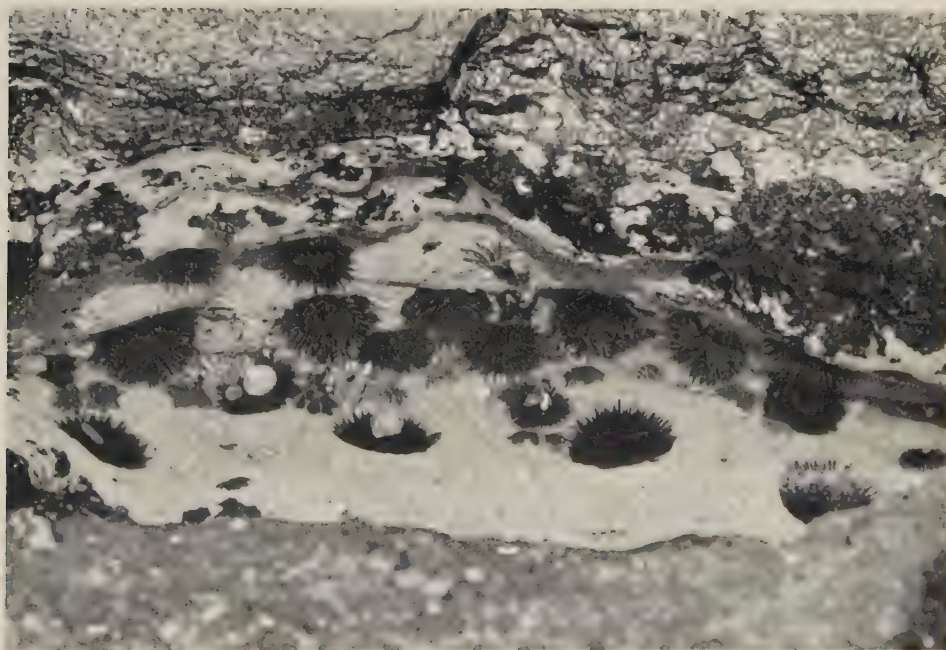


Fig. 2

DAKIN, BENNETT, and POPE.—A STUDY OF CERTAIN ASPECTS OF THE ECOLOGY OF THE
INTERTIDAL ZONE OF THE NEW SOUTH WALES COAST

The barnacle zones of the shores are well known and although the species are different and at times very characteristic of certain definite geographical regions, yet somewhat similar forms live under similar conditions on shores far apart. In fact, Ricketts and Calvin (1939) figure a *Tetraclita rubescens*—a dull brick-red barnacle—as occupying the middle shore zone and being a surf swept animal. We have our pink *Tetraclita rosea* in similar positions.

One of the most surprising examples is the spider of the surf zone, *Desis*. Species occur on shores from South Africa to Victoria and a series of these is found under the same puzzling conditions on each coast.

One could extend the list easily. We would therefore draw attention to the occupation of similar ecological niches on distant shores by similar types and stress the similarity in the basic zonation of shores separated by oceans and continents.

Much emphasis has been placed on the sublittoral and littoral area as a “cradle of evolution” and perhaps on a landward migration of species from the sea. One must never forget the geographical distribution of shore species *along* the world’s coasts. And one might venture to stress the view that through the vicissitudes of phylogenetic history the genus or family is often closely anchored to a specially favoured ecological niche, notwithstanding the workings of geographical distribution and isolation.

Shore faunas have extended themselves along the world’s coasts even if carried as larval stages by sea currents. Isolation has worked in some cases to produce highly local species. Naturally, in isolated regions, there may be specialities and in some cases entirely different types may be evolved to fit into ecological niches occupied otherwise elsewhere. One sees this on land in studies of the world’s floras. But the striking general resemblances remain.

The sensitivity of a seaweed like *Sarcophycus*, which seems to distinguish a difference between the outer ocean coast and the adjacent and apparently almost equally exposed rocks in the wide open Twofold Bay is remarkable. There are many animal cases of the same nature, some examples of which have been indicated.

There are some fascinating physiological and biochemical problems to be solved before the adaptations of seashore animals to their special zones are even partly understood (see Colman 1933, 1941; Stephenson *et al.* 1943). But this is very much in the realm of pure science and the answers are not likely to be forthcoming in the near future with other fields of research offering so much that is essential and remunerative today.

8. ACKNOWLEDGMENTS

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We also wish to thank Dr. A. B. Hastings of the British Museum (Natural History) for identifications of Bryozoa and Mrs. Valerie Jones of the National Herbarium and C.S.I.R. for her assistance in the determination of the algae.

9. REFERENCES*

* The literature noted below is referred to in the text. A very long list of taxonomic papers could be drawn up, since much systematic investigation has been called for in the research. Only a few references to papers of special taxonomic significance have, however, been included, since the aim of the investigation has not been systematic.

- AUGENER, H. (1914-1930).—Polychaeta II. Sedentaria. Michaelsen u. Hartmeyer—Die Fauna Südwest Australiens, Vol. 5: 1-172.
- AUGENER, H. (1927).—Polychaeten von Südost und Süd Australien. Papers from Dr. Mortensen's Pacific Expedition 1914-16, Vol. 38, *Vidensk. Medd. Dansk Naturh. Foren.* 83: 71-275.
- BROWN, IDA A. (1925).—Notes on the occurrence of glendonites and glacial erratics in upper marine beds at Ulladulla, N.S.W. *Proc. Linn. Soc. N.S.W.* 50: 25-31.
- BROWN, IDA A. (1928).—The geology of the south coast of N.S.W. Pt. I. *Ibid.* 53: 151-92.
- BROWN, IDA A. (1930a).—Pt. II. *Ibid.* 55: 145-58.
- BROWN, IDA A. (1930b).—Pt. III. *Ibid.* 55: 637-98.
- CLARK, H. L. (1946).—The echinoderm fauna of Australia and its origin. Carnegie Inst. Wash. Publ. No. 566.
- CLEMENTS, F. E., and SHELFORD, V. E. (1939).—"Bio-Ecology." (John Wiley & Sons: New York and London.)
- COLMAN, J. (1933).—The nature of the inter-tidal zonation of plants and animals. *J. Mar. Biol. Assoc. U.K.* 18: 435-76.
- COLMAN, J. (1941-42).—Some inter-tidal enigmas. *Proc. Linn. Soc. Lond.*, 154th Session, Pt. 3, June: 232-4.
- COTTON, L. A. (1947).—The pulse of the Pacific. *J. Roy. Soc. N.S.W.* 80(2): 41-76.
- DAKIN, W. J., and COLEFAX, A. N. (1940).—The plankton of the Australian coastal waters off New South Wales. Univ. Sydney, Dept. Zool., Monogr. No. 1.
- FAUVEL, P. (1917).—Annélides polychètes de l'Australie Méridionale. *Arch. Zool. Exp. Gén.* 56: 159-277.
- GISLÉN, TORSTEN (1930).—Epibioses of the Gullmar Fjord. A study of marine sociology. No. 3. Kristinebergs Zool. Stat. (1877-1927). Skrift. utg. av k. Svenska vetenskapsakad. Uppsala, 1-503.
- GULLIVER, F. P. (1898-9).—Shoreline topography. *Proc. Amer. Acad. Arts Sci.* 34: 151-255.
- HADDON, A. C., and DUERDEN, J. E. (1896).—On some Actiniaria from Australia and other districts. *Sci. Trans. R. Dublin Soc.* 6(2): 139-72.
- HALLIGAN, G. H. (1928).—The tides on the Australian coast. *Proc. Pan-Pacif. Sci. Congr., Aust.* (1928) 1: 214.
- HEDLEY, C. (1904).—The effect of the Bassian Isthmus on the marine fauna. *Proc. Linn. Soc. N.S.W.* 28: 876-83.
- HEDLEY, C. (1915).—Presidential Address. An ecological sketch of the Sydney beaches. *J. Roy. Soc. N.S.W.* 49: 1-77.

- HEDLEY, C. (1917).—A check-list of the marine fauna of New South Wales. Pt. I, Mollusca. *Ibid.* 51, Suppl. (1917): M. 1-120.
- HEDLEY, C. (1924).—Differential elevation near Sydney. *Ibid.* 58: 61-6.
- HEWATT, W. G. (1937).—Ecological studies on selected marine inter-tidal communities of Monterey Bay, California, *Amer. Midl. Nat.* 18(2): 161-206.
- JARDINE, F. (1925).—The development and significance of benches in the littoral of eastern Australia. Rep. Gt. Barrier Reef Cttee., *Trans. Roy. Geogr. Soc. Aust.* (Qd.) 1: 111-30.
- JOHNSON, D. W. (1919).—"Shore Processes and Shoreline Development." (Wiley: New York.)
- JOHNSTON, T. HARVEY (1917).—Presidential Address. Ecological notes on the littoral fauna and flora of Caloundra, Queensland. *Qd. Nat.* 2: 53-63.
- JUTSON, J. T. (1939).—Shore platforms near Sydney, New South Wales. *J. Geomorph.* 2: 237-50.
- MAY, VALERIE (1938).—A key to the marine algae of New South Wales. Pt. I. Chlorophyceae. *Proc. Linn. Soc. N.S.W.* 63: 207-18.
- MAY, VALERIE (1939).—Pt. II. Melanophyceae (Phaeophyceae). *Ibid.* 64: 191-215.
- OLIVER, W. R. B. (1923).—Marine littoral plant and animal communities in New Zealand. *Trans. N.Z. Inst.* 54: 496-545.
- POCOCK, R. I. (1902).—On spiders of the marine genus, *Desis*. *Proc. Zool. Soc. Lond.* 2(1): 104.
- POPE, E. C. (1943).—Animal and plant communities of the coastal rock platform at Long Reef, N.S.W. *Proc. Linn. Soc. N.S.W.* 68(5-6): 221-54.
- RICKETTS, E. F., and CALVIN, J. (1939).—"Between Pacific Tides." (Stanford Univ. Press: California.)
- ROBSON, C. H. (1877).—Notes on a marine spider found at Cape Campbell. *Trans. N.Z. Inst.* 10: 299-300.
- STEERS, J. A. (1929).—The Queensland coast and the Great Barrier Reefs. *Geogr. J.* 74: 232-57; 341-70.
- STEPHENSON, T. A. (1936).—The marine ecology of the South African coasts, with special reference to the habits of limpets. *Proc. Linn. Soc. Lond.* 148th Session: 74-9.
- STEPHENSON, T. A. (1939).—The constitution of the inter-tidal fauna and flora of South Africa. Pt. I. *J. Linn. Soc. Lond., Zool.* 40: 487-536.
- STEPHENSON, T. A. (1941-42).—Causes of vertical and horizontal distribution of organisms between tidemarks in South Africa. *Proc. Linn. Soc. Lond.* 154th Session, Pt. 3: 219-32.
- STEPHENSON, T. A. (1944).—The constitution of the inter-tidal fauna and flora of South Africa. Pt. II. *Ann. Natal Mus.* 10: 261-358.
- STEPHENSON, T. A. (1947).—Pt. III. *Ibid.* 11(2): 207-324.
- STEPHENSON, T. A., COLMAN, J., DELF, E. M., and CHAPMAN, V. J. (1943).—A symposium on inter-tidal zonation of animals and plants. *Proc. Linn. Soc. Lond.* 154th Session (1941-42), Pt. 3: 219-53.
- STEPHENSON, T. A., STEPHENSON, ANNE, TANDY, G., and SPENDER, M. (1931).—The structure and ecology of Low Isles and other reefs. *Brit. Mus. (Nat. Hist.) Gt. Barrier Reef Exped. Sci. Rep.* 3: 17-112.
- VOISEY, A. H. (1935).—The physiography of the middle north coast district of New South Wales. *J. Roy. Soc. N.S.W.* 68: 88-103.
- WENTWORTH, C. K. (1938).—Marine bench-forming processes. Water-level weathering. *J. Geomorph.* 1: 6-32.
- WHITELEGGE, T. (1889).—List of the marine and fresh water invertebrate fauna of Port Jackson and neighbourhood. *J. Roy. Soc. N.S.W.* 23: 163-323.

EXPLANATION OF PLATES 1-9*

(All photographs, with the exception of Plate 6, Fig. 2, by W. J. Dakin.)

* All photographs of rock platforms were taken at low-water spring tide.

Plate 1

Fig. 1.—Typical intertidal rock platform. (Southern side of Mona Vale Headland.)

Fig. 2.—Rock platform, Coalcliff. Most of surface above high-water mark.

Plate 2

Fig. 1.—*Hormosira Banksii* growth on part of Long Reef platform.

Fig. 2.—Characteristic growth of *Hormosira* around rim of rock pool.

Plate 3

Fig. 1.—Exposed rock platform at Harbord, Sydney, on day with calm sea, but moderate swell.

Fig. 2.—View along littoral-sublittoral fringe at extreme low water, showing algae *Phyllospora comosa* and *Ecklonia radiata*, with *Pyura praeputialis*. (Warden's Head, Ulladulla.)

Plate 4

Fig. 1.—Kelp zone at extreme eastern edge of Long Reef.

Fig. 2.—Zonation with barnacle, *Galeolaria*, *Pyura*, and kelp zones showing. (Long Reef, extreme eastern margin.)

Plate 5

Fig. 1.—*Pyura* zone. Extensive beds of this ascidian exposed at low water. (Warden's Head, Ulladulla.)

Fig. 2.—Exceptional development of encrustations of polychaete worm, *Galeolaria caespitosa* Lam. (Merewether, N.S.W.)

Plate 6

Fig. 1.—Example of sharp limits to zones. Margin of *Galeolaria* and barnacle zones on vertical wall of rock at Black Head, Gerroa. Barnacles are *Catophragmus polymerus* and *Tetracrita rosea*.

Fig. 2.—*Galeolaria* tubes separated to show sea spider, *Desis crosslandi* and web. Enlarged x 5. (Photo: Gwen Burns.)

Plate 7

Fig. 1.—Barnacle zone—*Chamaesipho columna* band along southern shore of Black Head, Gerroa.

Fig. 2.—A vertical face on rock platform, Harbord, showing surf barnacle zone (*Catophragmus* and *Tetracrita*) above *Galeolaria* zone.

Plate 8

Fig. 1.—Exposed slope on Harbord rock platform. In such splashed positions the surf barnacle zone is broadened. (Barnacles seen are *Tetracrita* with *Catophragmus*.)

Fig. 2.—The burrowing echinoderm, *Helicoidaris erythrogramma*, in typical hollows at low tide levels.

Plate 9

Fig. 1.—Zonation up the Hawkesbury River Estuary. The commercial oyster (*Saxostrea commercialis*) is seen above the alga *Hormosira* and below this is a band of mussels.

Fig. 2.—A "carpet" formed by the alga *Corallina* with evenly distributed anemones (*Anthopleura muscosa* (Drayt.)).

SIMULIIDAE (DIPTERA) FROM QUEENSLAND

By M. JOSEPHINE MACKERRAS* and I. M. MACKERRAS†

(Plates 1-2)

[Manuscript received December 17, 1947]

Summary

Eight species and one subspecies of Simuliidae are now known from Queensland, five of which are new. The life-histories of eight are recorded in this paper.

Only two of the species are known to attack man and domestic animals, and only one is a serious pest. This has been separated from *A. bancrofti* (Tayl.), with which it had been confused, and described as *A. pestilens* n.sp.

A. pestilens breeds in fast, turbulent, muddy water during the height of the floods in inland streams. It is predominantly associated with *Melaleuca* spp. in the stream beds, the early stages attaching to the submerged fronds, and the adults congregating in the exposed parts of the trees.

This and other inland species must possess a drought resistant stage, probably the egg.

Keys and figures are given for identification of adults, pupae, cocoons, and larvae.

I. INTRODUCTION

Prior to the present investigation, little was known of the Queensland Simuliidae. Taylor (1918) had described *Simulium bancrofti* Tayl., and this was the only Queensland species recorded by Tonnoir (1925), who placed it in the genus *Austrosimulium*, which he created for Australasian species with ten-segmented antennae. Taylor (1927) described *Simulium faheyi* on a single specimen from Innisfail, and subsequently (though on dubious grounds) recorded *Simulium ornatipes* Skuse as a pest in south Queensland (Taylor 1944). The pest species was generally known as *A. bancrofti* (Tayl.).

Nothing was known of the biology of the Queensland forms, but Mr. D. J. Lee (personal communication) had found the early stages of *S. ornatipes* in central Queensland, while Tonnoir (unpublished data) had worked out the life-histories of *A. bancrofti* and *S. ornatipes* in streams near Canberra, and Drummond (1931) had published an account of the same two species from Western Australia. Roberts (1940) has given some notes on the damage caused to stock by the pest species.

This work was undertaken primarily to elucidate the life-history of the pest species and to establish its identity, since somewhat conflicting statements had appeared about its morphology. The uncertainty proved to be due to the confusion of two species with very similar adults, which are quite distinct in the larval and pupal stages, and also have different habits in the adult stage. In all, eight species (one *Cnephia*, three *Simulium*, and four *Austrosimulium*) have been taken in south Queensland, and description of the adults, pupae, and larvae are given below.

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II. MATERIALS AND METHODS

During the period February to June 1947, many creeks and rivers were visited, and abundant material was usually found in those portions of the streams where there was a steady flow of water. Larvae and pupae were transported to the laboratory in 1-quart "Agee" jars, or in large enamel basins. They were found to stand motor transport fairly well, especially when they were placed with water weeds in large, shallow basins with a relatively small amount of water. The larvae of some species survived journeys lasting up to 7 hours. The jolting and consequent movement of the water was apparently beneficial in promoting aeration. Pupae were more easily handled in jars without water, the wet leaves or stones to which they were attached serving to keep the atmosphere fairly moist. Development and emergence proceeded normally under these conditions for at least 3 days in summer and 6 days in winter.

Adults of both sexes were taken in a muslin net by sweeping bushes along the banks. This method was not very productive, except for *A. pestilens*, which, during its short periods of abundance, clustered so thickly on the leaves of the *Melaleuca* and other shrubs that many hundreds could be captured with each sweep of the net.

Females were also taken ovipositing on rocks, logs, or other objects just awash. All collected in this way proved to be *S. ornatipes*.

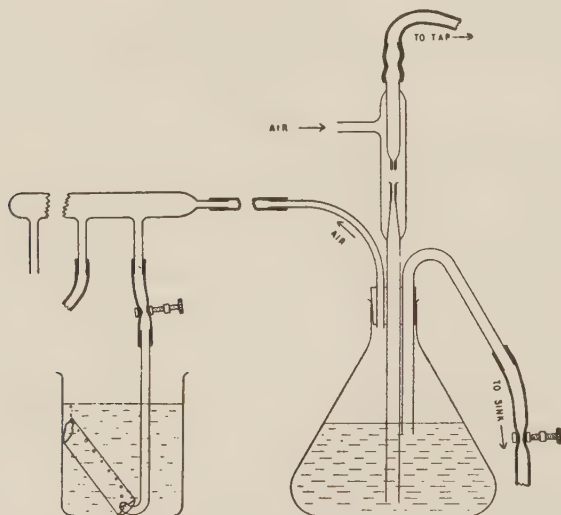


Fig. 1.—Diagram of the equipment used for breeding Simuliidae in the laboratory.

In the laboratory, aeration was provided by a reversed suction apparatus, designed and made by D. Mackerras, and known conveniently as "the bubbler" (Fig. 1). A powerful air pressure was developed in this apparatus, sufficient to bubble air rapidly through at least eight jars. A short, wide, glass cylinder, open at both ends, was placed obliquely in each breeding jar, and the

stream of bubbles directed into it. This device created a current of water, and larvae rapidly found the optimum conditions and arranged themselves inside the cylinder on each side of the stream of bubbles. Other favoured sites were the edge of the cylinder, both top and bottom, and the sides of the glass breeding jar where the bubbles impinged. Small quantities of water were removed daily, and replaced by water from an aquarium containing much microscopic life.

Two species, *S. ornatipes* and *S. nicholsoni*, did fairly well under these artificial conditions, and the former was reared from egg to adult in the laboratory. Other species were not so adaptable; well-grown larvae usually pupated successfully, but small larvae invariably died.

Adults were removed from breeding jars by a suction apparatus, and kept for several hours or days before being killed and pinned. Some survived for 5 days on a diet of raisins or apple.

The descriptions are based on a variety of material, but have been checked in every instance from single bred series; the adults were always described as soon as they were killed, and before shrinkage or other changes could occur. The females are described before the males, and have been selected as the holotypes of the new species, because they are more easily recognized.

The three genera (*Cnephia* End., *Simulium* Latr., and *Austrosimulium* Tonn.) in which the Australian species have been placed by Smart (1945), following Edwards (1931), are accepted here provisionally, a discussion of their status being reserved for another paper. Mention may be made, however, of the occurrence within the genus *Simulium* of an interesting group of species which have a conspicuous patch of bright, creamy or golden scales on the membranous prealar area. They are also distinguished by possessing numerous small, but easily seen, hairs on the "mesosternal" area. This group seems to be essentially northern in distribution, extends to New Guinea (R. H. Wharton), and is probably related to Edwards' (1934) Sub-group C from the Netherlands East Indies. Though the adults are homogeneous, the pupae are diversified, and it is difficult to say whether it is a natural assemblage or not.

III. GENERAL BIOLOGY

The abundance and wide distribution of several species in Queensland has been surprising, and that they have attracted so little attention is evidently due to the fact that the majority of species do not attack man or domestic animals, the adults being extremely inconspicuous.

Only two species have been taken biting man at all frequently, namely *A. bancrofti* (Tayl.) and *A. pestilens* n.sp. *A. bancrofti* has occasionally been taken biting horses in Queensland and man in the Australian Capital Territory, and Drummond (1931) reports that it bites viciously in the Darling Ranges, Western Australia, particularly in spring and summer. In Queensland it is certainly not troublesome, whereas *A. pestilens* attacks man and domestic animals viciously over a wide area of country. Fortunately it has only a short season each year, and then apparently disappears entirely for long periods.

Of the remaining species, only *S. nicholsoni* took human blood occasionally. This, to judge by the prevalence of larvae, is an abundant species, but the even more abundant *S. ornatipes* has never been seen to bite at all. Taylor's (1944) record of it as a pest would appear to have been a misidentification. We have no idea what these and the rarer species feed on; it is certainly not man nor domesticated animals, and there is a little experimental evidence to suggest that *S. ornatipes* may be able to mature eggs without a feed of blood at all.

The normal resting places of the adults are also obscure. Tonnoir (1925) had some success with southern and New Zealand species by sweeping the bushes along the streams where the early stages were found, and we have mentioned the association of *A. pestilens* with *Melaleuca* in the creek beds. In a search of bushes and other likely and unlikely situations near breeding grounds, we have found a few *S. ornatipes*, *S. nicholsoni*, and *A. bancrofti*, but the numbers taken were meagre in the extreme as compared with the abundance of larvae and pupae nearby. Their haunts are either very cryptic, or dispersal is immediate and wide. The latter view was held by Taylor (1944), and is supported by finding *A. bancrofti* in brigalow scrubs at Eidsvold at considerable distances from running water (T. L. Bancroft).

The early stages occurred in a wide variety of situations, and were not restricted to permanent water, nor to mountain streams; in fact, they were more prevalent at low than at higher altitudes. After the wet season, *S. ornatipes* colonized many little, temporary streams, and even roadside gutters, while there was a steady flow of water. All the species studied were facultatively herbicolous or lapidicolous. *S. clathrinum*, *A. furiosum*, and *A. mirabile* were only found in perfectly clear water; *S. ornatipes* also seemed to prefer clear water; but *S. nicholsoni* and *A. bancrofti* tolerated considerable degrees of muddiness, while *A. pestilens* was only found in very muddy streams.

The species also showed definite preferences in the speed of water in which the larvae lived. Of the coastal species breeding in clear water, *S. clathrinum* and *A. bancrofti* preferred the fastest, *S. nicholsoni* not so fast and with a wider range, and *S. ornatipes* the slowest. Inland, where the gradient varies little and all streams are senile, this relation to speed of water (and perhaps also to muddiness) resulted in a definite succession of species following the floods. While the creeks and rivers were in flood, *A. pestilens* bred in almost pure culture, thriving in the turbulent, muddy water, which seemed unsuitable to other species. As the rate of flow diminished and the water cleared somewhat, other species appeared, and displaced *A. pestilens* entirely. *A. bancrofti* and *S. nicholsoni* thrived at this stage, and to a lesser extent *S. ornatipes*, which, however, alone remained when the stream was reduced to a mere trickle.

How these inland species survive the long, dry summer, when all water-courses cease to flow is at present a mystery. Larvae die rapidly when removed from water, and it seems unlikely that pupae could survive the heat and dryness associated with a dry creek bed. Among the countless pupal shells left by the

receding flood, there is always an appreciable number containing unemerged adults. Most of those recently exposed probably emerge (as they do in the laboratory), but those long exposed appear to be completely desiccated, and showed no sign of reviving when placed in the "bubbler." The adults appear to be short-lived, and the most plausible theory seems to be that there is a resistant egg stage which tides the organism over to the next wet season. We have so far experimented only with *S. ornatipes*, and have not yet succeeded in hatching dried eggs in the laboratory.

IV. KEYS TO SPECIES OF SIMULIIDAE

(a) Adults

1. Antennae 11-segmented 2
 Antennae 10- (sometimes 9-) segmented (*Austrosimulium*) 5
2. Large species with strongly humped, orange scutum, fawn to yellowish fore legs and antennae *Cnephia tonnoiri fuscoflava* n. subsp.
 Smaller, darker species; scutum of normal form and with dark integument; neither fore legs nor antennae entirely pale (*Simulium*) 3
3. Prealar area bare; legs banded conspicuously with creamy-yellow *S. ornatipes* Sk.
 Prealar area with conspicuous pale scales; legs not as above 4
4. Frons of female one-seventh of head width, abdominal segments 2 to 9 entirely black dorsally; male with upper eye facets more enlarged than usual; scutum in both sexes with indefinite golden median and dorso-central lines, and veins of wing-root very dark *S. clathrinum* n.sp.
 Frons of female one-fourth of head width, at least tergites 5 to 8 of the abdomen with conspicuous pale lanceolate scales dorsally; male with upper eye facets normally enlarged; scutum in both sexes without trace of median and dorsocentral lines, and veins of wing-root yellow to brownish *S. nicholsoni* n.sp.*
5. Wings with three dark spots behind costa; antennae long, with fourth to sixth segments orange *A. mirabile* n.sp.
 Wings unspotted; at most basal two segments of antenna paler than rest 6
6. Abdomen entirely dark dorsally *A. furiosum* (Sk.)
 Abdomen of female with median and sublateral ashy patches dorsally; of male with shiny, ashy, sublateral patches which are visible in dorsal view 7
7. Antennae 10-segmented; third segment in female little larger than second *A. pestilens* n.sp.
 Antennae 9-segmented; third segment in female markedly larger than second *A. bancrofti* (Tayl.)

* The unique female of *S. faheyi* Tayl. would run nearest to *S. nicholsoni* in the key, but it is distinguished by its brown and gold rather than black and creamy coloration, and by possessing small teeth on the claws.

(b) *Early Stages*

Larvae old enough to show a pupal gill-spot and pupae may be recognized more easily from Figures 5 and 8 respectively than from a key. Larvae which do not show a gill-spot may be tentatively placed by means of the following key.

1. Anal sclerite without backwardly-directed strut (*Cnephia* and *Simulium*) 2
 Anal sclerite with backwardly-directed strut (*Austrosimulium*) 5
2. Ventral papillae absent 3
 Ventral papillae present (rectal gills simple) 4
3. Rectal gills compound *S. clathrinum* n.sp.
 Rectal gills simple *C. tonnoiri fuscoflava* n. subsp.
4. Head pattern "positive" type*; robust, dark species *S. ornatipes* Sk.
 Head pattern "negative" type*; more delicate, yellowish species *S. nicholsoni* n.sp.
5. Ventral papillae present 6
 Ventral papillae absent 7
6. Chitinous rod encircling tip of abdomen ventral to anal sclerite *A. mirabile* n.sp.
 No such rod present *A. furiosum* (Sk.)
7. Anal sclerite stout, angle between anterior limbs usually less than 90°;
 submental teeth reduced to 7 *A. bancrofti* (Tayl.)
 Anal sclerite delicate, angle between anterior limbs usually greater than 90°;
 submental teeth 11 *A. pestilens* n.sp.

V. THE GENUS *CNEPHIA* END.*CNEPHIA TONNOIRI FUSCOFLAVA* n.subsp.

Simulium tonnoiri Drummond, 1931, p. 6. (typical subspecies)

Larva

The full grown larva measures 6.5 to 7 mm., and is robust, usually grey or brown in colour and distinctly darker towards the tip of the abdomen, which is particularly stout. Head capsule usually heavily pigmented, pattern on dorsum (when detectable) as in Figure 2c. Submentum and antennae as in Figures 2a and 2b; base of cephalic fans dark; ventral notch of head capsule wide and shallow.

Gill-spot (Fig. 2d) broad, irregularly L-shaped, with the horizontal limb directed backwards. The arrangement of the filaments is unusual, in that, having turned posteriorly once (forming the right-angle of the L), they fold back on themselves ventrally and anteriorly, so that the tips of the filaments come to lie along the anterior face of the vertical limb of the L. In the species of *Simulium* studied, the filaments are coiled posteriorly.

Rectal gills simple; anal armature as in Figures 2e and 2f; no ventral papillae, but slight ventrolateral swellings are present as in *S. clathrinum*. The posterior

* Edwards (1934) defines the markings of the head-capsule as "positive" when the insertions of the muscles are darker than the surrounding chitin, "negative" when they are paler.

circlet is armed with about 120 rows, each row containing about 15 spines, which are relatively long, so that the circlet appears wide.

Pupa

Length 3 to 3.5 mm. Head and thorax smooth; thoracic hairs scanty, rather stiff and curly. Gill filaments (Fig. 2g) branched, variable in number, usually 15 to 20 being present. There are three main trunks (dorsal, ventral, and lateral) arising from a very short common stem; these give rise to other branches, which in turn may branch again. Abdominal armature (Fig. 2h) distinctive in having numerous, well-developed, backwardly-directed, sub-basal spines on the dorsal surface of segments 5 to 9, and in possessing a pair of stout, terminal hooks, which curve upwards and forwards.

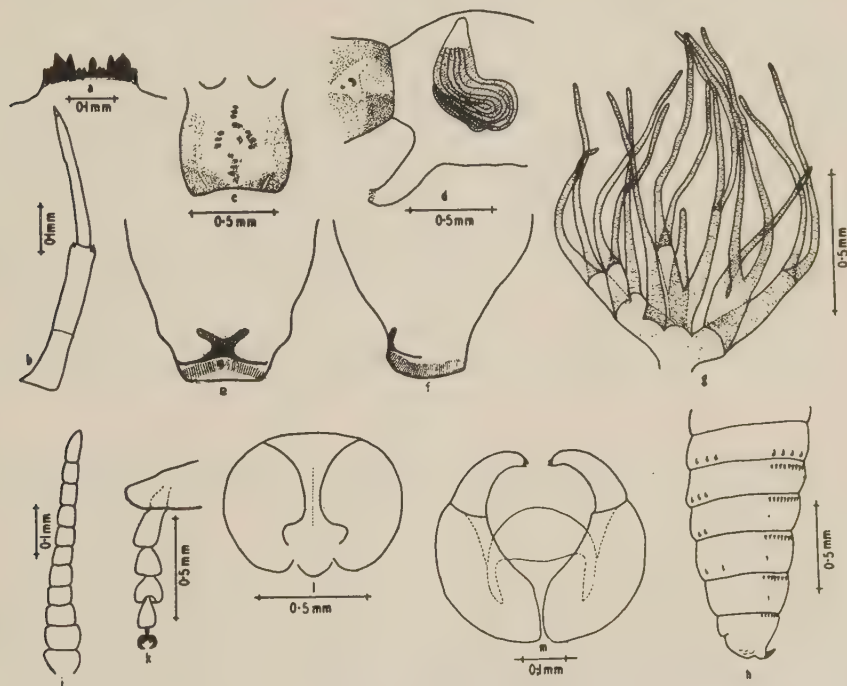


Fig. 2.—*Cnephia tonnoiri fusciflava* n. subsp.

Larva: a. submentum; b. antenna; c. head (dorsal view); d. gill-spot; e. dorsal view of anal armature; f. same, lateral view. Pupa: g. gill filaments; h. lateral view of abdominal armature. Adult: i. antenna of female; k. hind tarsus of female; l. head of female; m. hypopygium of male.

Cocoon

Tough, coarsely woven, with foreign matter incorporated in it; soft, and completely lacking the precise form and clear outlines of the cocoons of other Queensland Simuliidae. In shape it is a narrow bag, with a pointed, V-shaped closed end, and an irregular opening, through which more or less of the gill filaments project. The cocoons are frequently obscured by filamentous algae.

Female

Length: 3 to 3.5 mm., wing 2.5 to 2.8 mm.

Head: Frons dark grey, about one-tenth of head width at narrowest point (Fig. 2l), with fine, pale golden hairs, which become denser and longer towards the occiput; face dark grey, with pale golden hairs. Antennae (Fig. 2i) almost uniformly fawn coloured, perhaps slightly darker towards base of third segment, with coarser hairs on the basal three to four segments than in other Queensland species. Palpi and mouth-parts black.

Thorax: Scutum strongly arched, orange, covered with fine, pale golden hairs, which are denser in front of scutellum, which is also orange. Pleurae fawn coloured, anterior spiracles ringed with black, mesosternum dark grey. There is the upper mesepimeral hair tuft seen in all species, but no propleural, prealar, or mesosternal hairs.

Wings: With yellowish veins and two dark spots, one around the transverse vein at the root of the wing, and the other at the fork of R (and including r-m). Chaetotaxy normal, with a single row of long macrotrichia on the upper surface of the distal part of Rs. Distinct spiniform macrotrichia on costa but not on R₁. Cu₁ gently sinuous. Halteres uniformly creamy-fawn.

Legs: Fore legs fawn, darkening towards tips of tarsal segments; mid legs fawn, suffused with dark grey; hind legs almost entirely dark grey, but with an underlying brownish suffusion. Calcipala very large, blackish; pedisulcus small but quite distinct; claws with a strong basal tooth (Fig. 2k).

Abdomen: Dark, greyish-brown, covered fairly densely with pale creamy-gold hairs. Genital fork similar to the species of *Simulium*.

Male

Slightly larger than female, but otherwise similar, except for the large, holoptic eyes, darker mid and hind legs, and almost black ground colour of the abdomen. Hypopygium as in Figure 2m.

Taxonomic Notes

Holotype female, allotype male, and morphotype pupa and larva, from Dunwich, Stradbroke Island, S. Queensland, 10.x.47, in collection of the Division of Economic Entomology, C.S.I.R., Canberra.

This species was discovered by Miss E. N. Marks after the body of the paper had been completed and the figures prepared for publication. It has therefore been necessary to treat it separately, which is not inappropriate as it stands widely apart from other known Queensland members of the family. It is closely related to *C. aurantiacum* (Tonn.) from south-eastern Australia and Tasmania, but still nearer to *C. tonnoiri* (Drum.) from Western Australia. From the former, it is to be distinguished in the adult by lacking propleural hairs and possessing a well-defined dark spot on the wing, in the pupa by its markedly coarser, less numerous gill filaments, and in the larva by its somewhat narrower gill spot (cf. Fig 6A in Tonnoir 1925) and larger, less numerous teeth in the posterior circlet (about 15 as compared with 30). Its differences from typical *C. tonnoiri* are minor but apparently constant. The adults are distinctly darker, especially the mid and

hind legs, but we could find no structural differences; the gill filaments of the pupae are a little coarser and fewer (15 to 20 as compared with 20 to 30 in the typical form); and the gill-spot of the larva is somewhat broader, and the teeth of the posterior circlet somewhat fewer (18-24 in typical *C. tonnoiri*). Incidentally, the cocoons of *C. aurantiacum* often show some approach to a definite form, whereas those of the other two are little more than shapeless bags.

The presence of propleural hairs in the adults of *C. aurantiacum* is a definite specific character, but no such clear distinction could be found between our material and typical *S. tonnoiri*, of which, thanks to Dr. A. J. Nicholson, we had a good series for comparison. Nevertheless, we can distinguish consistently between the two, and so feel that subspecific rank would best indicate their relationship.

Biology

Eggs were not found, but larvae of all ages were abundant in clear, moderately fast, somewhat peaty water flowing in a narrow, man-made channel, and in a small, sunlit, natural stream on the narrow coastal flat just before it ran out onto the beach. They were attached to vegetation, to submerged sticks and logs, and especially to long grass blades just beneath the surface. Pupae were found in the same situations, crowded together, often overlapping one another, and frequently so matted with filamentous algae as to be difficult to detect. Pupae kept out of water in moist jars continued to emerge for 3 to 4 days after being collected.

Adults were not observed in the vicinity of their breeding places, and none was taken by sweeping. This species has not been recorded as attacking man or domestic animals in the nearby settlement.

Distribution

Dunwich, Stradbroke Island, 28.ix.47 (E. N. Marks), 10.x.47 (authors).

VI. THE GENUS *SIMULIUM* latr.

SIMULIUM ORNATIPES Skuse

Simulium ornatipes Skuse, 1890, p. 595; Tonnoir, 1925, p. 232; Drummond, 1931, p. 6; Taylor, 1944, p. 213.

Egg

Pear-shaped, approximately 0.2 mm. by 0.13 mm., laid side by side, with long axis at right angles to the substrate and the narrower end pointing upwards. The eggs are glued securely together and to the leaf or stone on which they are deposited.

Larva

Length of full-grown specimens 6 to 6.5 mm. Body creamy, grey, or greenish, irregularly mottled with darker patches. Melanic individuals are frequent. Pattern on dorsum of head variable, often as in Figure 3. Antenna and submentum as in Figure 4a. Gill spot large, black, conspicuous (Fig. 5). Rectal gills consist of three simple digitations. Anal armature as in Figure 6. Ventral papillae present. The posterior circlet ("sucker") is armed with about 60 to 70 rows of spines, 10-20 spines per row.

Pupa

Length 2.5 to 3 mm. Head covered fairly evenly with small flat tubercles, cephalic hairs fine and short; thorax similarly covered, thoracic hairs fine, simple, inconspicuous. On the dorsum of the abdomen, the second segment bears a row of hairs, the third and fourth each carry 4 forwardly-directed strong hooks on each side of the midline, the seventh and eighth segments have 5 or more backwardly-directed, flat, sub-basal spines on each side of the midline, and the terminal segment bears a pair of small, strong hooks. On the ventral surface, the fourth segment bears a pair of forwardly-directed hooks, the fifth, sixth, and seventh each 2 pairs, those on the fifth segment being placed close to the midline, whilst the others are more or less widely spaced; the hooks are usually bifid.

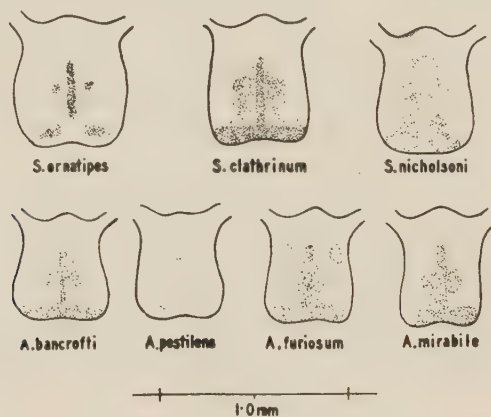


Fig. 3.—Larvae. Dorsal view of heads.

The respiratory organ on each side consists of a short wide stem, from which spring four long, wide, thin-walled tubes or filaments. These filaments are arranged in a characteristic way in life, one limb of the external pair being directed upwards and inwards and crossing its fellow from the opposite side, and the other limb, which is the longest of the four, pointing directly forwards. The limbs of the inner pair are directed upwards and forwards.

Two forms of *S. ornatipes* have been observed, which differ only in the length of the gill filaments. In one, the filaments taper gradually to a blunt point, and measure 2.5 to 3.0 mm.; in the other form they are short, wide tubes which measure 1.4 to 2.0 mm. (Fig. 7). Both forms vary considerably, but intermediates difficult to place in either category are uncommon. The two forms have been taken together in many localities, often on the same blade of grass, but we have occasionally taken only one of them. For example, in a small stream at Brookfield, only "long horned" forms were found at numerous examinations from February to June; whereas, in fast running water in Heifer Creek in March, only "short horned" forms occurred (one examination). We have the impression that the "long horned" forms are better adapted for survival when the flow of water

is reduced. It would clearly be unwise to give these forms any taxonomic status until a great deal more is known about them.

Cocoon

Wall-pocket type (Fig. 8), rather coarsely woven, with an irregular margin which projects anteriorly to a variable degree in the mid-dorsal line. The sides of the cocoon do not meet below the head of the pupa, in contrast to the cocoons of the Western Australian forms, which often have a distinct collar (Drummond 1931). The ventral wall of the cocoon is incomplete, only covering the abdomen of the pupa.

Female

Length: 2.7 mm. to 3.3 mm.; wing 2.1 mm.

Head: Frons narrow, one-seventh head width (Fig. 9), with greyish tomentum and unevenly distributed, white, lanceolate scales, which extend behind the occiput, where they become more golden. Face similar, but tends to be more silvery. Antennae (Fig. 10a) with first two segments brownish-yellow, remainder dark brown with short, shining, white hairs. Mouth-parts and palpi greyish-brown, with paler hairs.

Thorax: Scutum brownish-black, with inconstant traces of paler median and dorsocentral lines; covered for the most part with black lanceolate scales, and much more conspicuous white and golden ones, which are somewhat irregularly arranged. There is a zone of whitish scales above the lateral and anterior margins, irregular golden dorsocentral patches posteriorly, and a rather dense paler zone in front of scutellum. Scutellum black, with golden hairs; metanotum dark brown. Pleurae grey, without prealar patch of scales.

Wings: Clear; R with macrotrichia above. Halteres with knob and most of stem pale cream, base somewhat darker.

Legs: Fore coxae entirely cream, rather densely covered with whitish hairs anteriorly. Mid coxae cream, with more or less greyish suffusion. Posterior coxae grey, creamy towards tips. Femora creamy-yellow, with narrow blackish zone apically (very narrow on anterior legs, wider on posterior legs). Fore tibiae creamy-yellow, with darker zone at base and apically; mid tibiae with dark sub-basal area, creamy-yellow proximal half, and dark distal half; hind tibiae mainly dark, with a creamy-yellow band extending from one-quarter to half the tibial length from the base. Fore and mid metatarsi entirely dark, hind creamy-yellow on basal three-fourths, dark apically. Subsequent tarsal segments of all legs dark, except base of hind second tarsal which is pale. Calcipala, pedisulcus, and claws as in Figure 11a.

Abdomen: Brownish-black dorsally, light yellowish-brown ventrally, the two areas being divided about the mid-lateral line. First visible segment has usual fringe of pale hairs, and subsequent segments have zones of conspicuous large pale scales, which tend to form an indefinite pattern of a median stripe and transverse bands towards the apices of segments. Genital fork as in Figure 12a.

Male

Darker and more slender than female. Antennae with basal two segments yellow; remainder dark brown, fading towards tip. Proportions of segments much as in female, but third segment relatively slender (Fig. 10h). Mouth-parts and palpi darker than in the female. Scutum black, covered dorsally with black and dark bronzy lanceolate hairs. Lateral margins and whole of sublateral areas anteriorly with relatively dense, whitish, lanceolate scales, darkening to gold posteriorly; very indefinite dorsocentral and median lines of golden scales, and dense paler ones on front of scutellum. Wings with veins darker than in female; halteres with knob creamy-yellow, stem and base brown. Legs as in female, but pale portions a darker yellow.

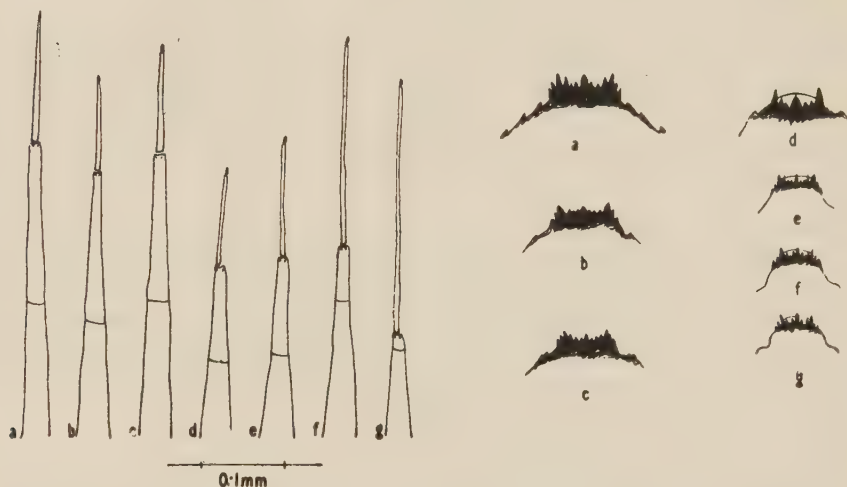


Fig. 4.—Larvae. Antennae (left) and submental teeth (right) of: a. *Simulium ornatipes* Sk.; b. *S. clathrinum* n.sp.; c. *S. nicholsoni* n.sp.; d. *Austrosimulium bancrofti* (Tayl.); e. *A. pestilens* n.sp.; f. *A. furiosum* (Sk.); g. *A. mirabile* n.sp.

Abdomen black dorsally, except for the second segment, which is extensively grey in the sublateral region. Venter pale brownish-yellow, with the dark colour of the dorsum tending to extend a little more ventrally on the posterior segments than in the female. Fringe of pale hairs on basal segment very conspicuous laterally, creamy at base, silvery towards the tip. Paler hairs on dorsum forming a very indefinite central golden stripe on most segments in some specimens, while in others the golden patches are more extensive and tend to form apical bands. Conspicuous pale gold to silvery patches on segments five to nine. Hypopygium as in Figure 13.

Taxonomic Notes

The description of all stages was based on specimens from Brookfield, near Brisbane. *S. ornatipes* is very distinctive in the Australian fauna by reason of its leg markings and pupal respiratory filaments, but it was thought wise to describe

the local form in some detail, as it is possible that subspecific differentiation may exist in different parts of the extensive range of the species.

Biology

We know nothing of the natural feeding habits or resting places of the adults, the only specimens taken in the field being ovipositing females. In the laboratory, the adults were strongly phototropic; they fed fairly readily on raisins or fresh fruit, but refused, in the few trials made, to take human blood. They survived up to five days in small jars, and considerable development of the ovaries occurred on the fruit diet. Over 150 follicles were present in each ovary.

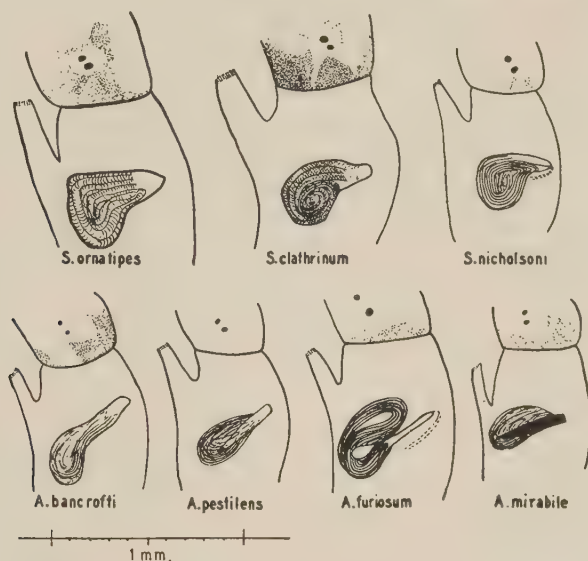


Fig. 5.—Larvae. Lateral view showing gill-spots.

The breeding grounds were widespread and varied, hardly a running stream from the New South Wales border to Rockhampton failing to harbour the early stages at one place or another. They were found most frequently alone in smaller, grassy streams with a moderate flow (Plate 1, Fig. 1); but they also occurred in faster water in company with *S. nicholsoni* or *S. clathrinum* and occasionally with *A. bancrofti*. At the other extreme, they were found in mere temporary trickles in road-side gutters. Always they were in fairly to quite clear water.

Females have been observed in several localities ovipositing on logs and rocks in a fairly fast current (Plate 1, Fig. 2). They were active in the morning about 9 a.m., and also in the afternoon, especially towards sundown. Sometimes only single individuals were seen, but more often there was a little cloud of perhaps a dozen or so apparently competing for the favoured spot. The females hovering a few inches above the water would settle, often two or three at a time, on the exposed surface of the object, and then back down until they could deposit their eggs at the very edge of the water. They were not seen deliberately to crawl

under the water. Females in the act of ovipositing clung tightly to their support, and were often not carried away even though a wave washed right over them. When they were washed off, or dislodged by other females, they would quickly return to the spot and soon settle again. The result is that each large sheet of eggs is a mosaic of the ovipositions of many females, each one contributing several small groups of eggs, separated from one another by groups laid by other females, and the whole cemented together into an even, uniform sheet. Sometimes a rock a foot in width would be ringed by a band of eggs perhaps two inches wide.

It is curious that throughout the work this is the only species that we saw ovipositing, and the only one of which we found the eggs.

On rocks and logs, the eggs were frequently found in the sheets described, though sometimes in small batches (about a centimetre in width) which may have been the product of a single female. On grass, on the other hand, they were generally in small, separate batches, but sometimes the patches were two or three centimetres long, again suggesting the work of more than one female. When freshly laid, the eggs were white, and they gradually developed a light brown shade, with dark brown tips. The brown colour hid them on rocks and logs, but made them conspicuous, easily found objects on grass.

In the laboratory, the duration of the egg stage was about 5 days in late summer, when the temperature of the water varied from 24° to 29°C.

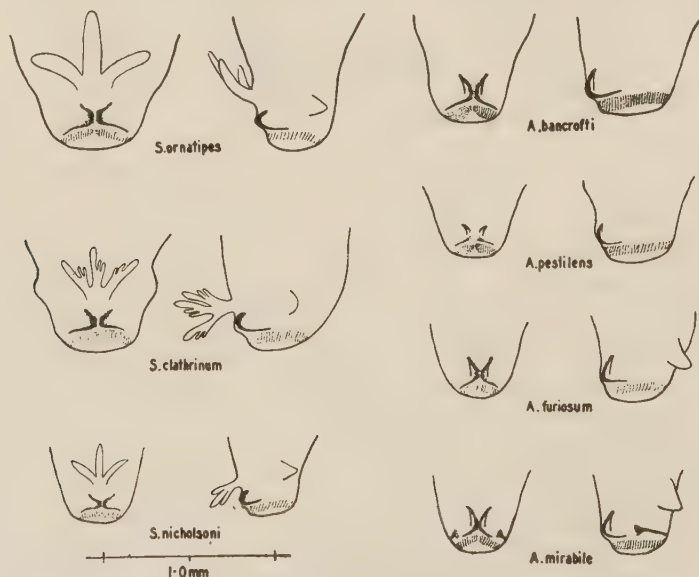


Fig. 6.—Larvae. Dorsal and lateral views of terminal segments.

In the field, the larvae occurred in all the situations mentioned above, and also in artesian bore drains at Cunnamulla (T. Greaves). They favoured clear water, but could tolerate suspended matter at least to the degree of opalescence.

They showed the typical *Simulium* habit of spacing themselves so that each one was a little apart from its neighbours, and they reacted to intruders by vigorous striking movements with their heads. Most commonly they were attached to blades of grass, or other vegetation, within an inch or so of the surface, but were also frequent on stones. As the water level fell, they would move downwards, and could sometimes be found on stones and dead leaves when there was only just sufficient water to cover them and the flow was reduced to a mere trickle.

Pupae were found with the larvae, but showed some tendency, though not as marked as with some other species, to concentrate in situations a little protected from the direct force of the current.

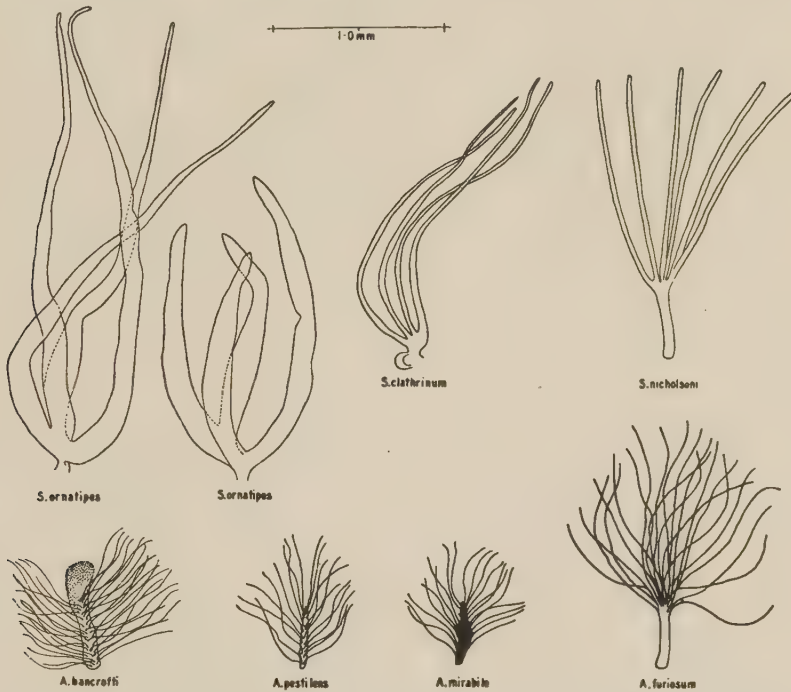


Fig. 7.—Pupae. Lateral view of breathing horns. Note: For the sake of clarity not all the gill filaments are shown in *A. bancrofti*, *A. pestilens*, and *A. mirabile*.

In the laboratory, some of the newly hatched larvae began feeding at once, others floated away still attached to the egg site by a silken strand issuing from their mouths. These strands became entangled in water weeds etc., and enabled the larvae to get a grip in a fresh place. They fastened themselves by their posterior circlet, and fed in exactly the same way as the large larvae. Larvae required a clean surface to fasten themselves to, and perished rapidly if the glass jars became slimy. The majority of these larvae required over 3 weeks for their development, the minimum larval periods recorded in two batches being 20 and 22 days. This is probably considerably in excess of what is required in the field

under ideal conditions. We strongly suspect that neither the food nor the composition of the water in the jars remained satisfactory for long. The really favourable situations, too, seemed to be limited, and very great variation in rate of growth was observed, some larvae being still tiny when others had reached full size.

When ready to pupate, larvae frequently moved about for some time, apparently seeking a suitable spot, but seldom moving out of the current. They usually began cocooning rather intermittently, and in the intervals of resting could be readily distinguished from feeding larvae by the position of their mouth brushes, which were not extended. They required about half an hour to complete the cocoon. The larva then let go its hold by its posterior circlet, and, lying transversely across the mouth of the cocoon, assumed a U-shape with the loop of the U within the cocoon. It then kept up a to-and-fro, semi-rotary movement, until the larval skin burst. This usually required from 3 to 10 minutes. As the pupa extracted itself from the larval skin, the abdomen was pushed down into the cocoon, and the thorax with expanding gill filaments filled the mouth of the cocoon. For several minutes the pupa continued to fidget about within the cocoon, sometimes rotating completely round. This movement served to entangle the abdominal hooks in loose strands of silk and so hold the pupa firmly in the cocoon. Meanwhile the gill filaments uncoiled and gradually took up their characteristic positions. Tonnoir (1923) has figured the process clearly in a New Zealand species, *Austrosimulium tillyardi*.

Newly formed pupae were pale yellow, but very rapidly the imaginal head with its reddish eyes became differentiated, the thoracic appendages became visible, and the whole insect darkened. On the third day, the pupa was very dark, and began to make intermittent, slow, writhing movements. A layer of air was then secreted under the pupal skin, giving it a silvery appearance. When the pupal skin split, the fly quickly freed itself from the old skin, and, still enclosed in its bubble of air, rose to the surface. Although the wings frequently contained some yellow fluid at this stage, the insect could usually take off successfully straight away from the surface of the water. The pupal period lasted 3 days in late summer at 24° to 27°C., and 7 days in winter, when the temperature of the water varied from 9° to 22°C. in the 24 hours.

Summarized, the developmental periods observed in summer were:

- egg — 5 days
- larval — 20 days (minimum)
- pupal — 3 days.

The first and the third are probably fairly accurate, the 3-day pupal period in particular being supported by numerous observations on pupating larvae collected in the field, and apparently being normal for several species. Judging by the growth of larvae of various ages collected in the field and set up fresh in the "bubbler," we would guess at the normal developmental period from oviposition to adult as between two and three weeks.

S. ornatipes is adapted to survive great reduction in flow, but it disappears when flow stops. Whether it survives in a few streams that continue to trickle; and recolonizes the others after rain, or whether it has a drought-resistant stage, we do not yet know. Eggs which had not been dried, hatched in still as well as in circulating water, but in the former the larvae quickly died; there was no evidence of suspended development in still water. Owing to the habit of the female of laying at the water edge, large sheets of eggs were often found in situations which would assuredly be dry next day, owing to the rapid fall in water level as the floods subsided. This suggested that the eggs might be resistant to drying, but dried eggs did not hatch when subsequently wetted, although they swelled and split, and dead larvae protruded from the openings.

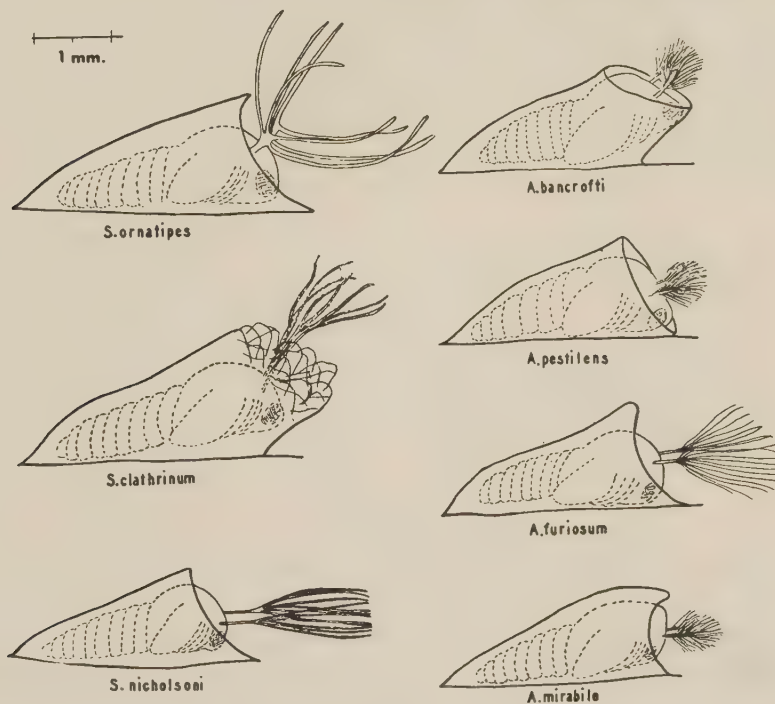


Fig. 8.—Cocoons. Lateral view.

Distribution

Brisbane district—Brookfield, February to June, absent from August to November; Moggill Ck., June 29; South coast—Mudgeeraba Ck., March and May; Springbrook, March; Mt. Tamborine, March; Samford district—Dawson Ck., April; South Pine R., April; Southern Tableland—Heifer Ck., March; Stanthorpe, March; Dalby—Back Ck., April; Chinchilla—Charley Ck., April; Cunnamulla, November 1946 (T. Greaves); Brisbane R., Wivenhoe, April; Blackbutt Range, April; Nanango, April; Kilcoy (Sheep Station Ck.), May; Goomeri (Barambah Ck.), May; Gayndah (Barambah Ck. and Reed's Ck.), May; Eidsvold (Burnett

R. and numerous creeks in district), April and May; Dawson R. (Camboon Woolshed, Theodore), April; Dee R. (Rannes), April; Don R. (Calliungal), April; Callide R. (Calliungal), April, May; Mt. Morgan, April; Rockhampton, April.

SIMULIUM CLATHRINUM n.sp.

Larva

Length 5.7 to 6 mm. Body greyish or greenish, irregularly mottled with darker areas. Head usually heavily pigmented, bases of the cephalic fans and mouth-parts all darker than in *S. ornatipes*. Pattern on the dorsum of the head variable; a common arrangement is shown in Figure 3. Submentum darker than in *S. ornatipes*, with 5 to 7 stout, dark spines on each side; arrangement of teeth as in Figure 4b; antennae as in Figure 4b (left). Gill spot rather small, pear-shaped (Fig. 5). Rectal gills compound, each of the three main divisions subdivided into three, occasionally four, unequal processes (Fig. 6). Anal armature as in Figure 6. Ventral papillae absent. Posterior circlet consists of about 90-95 rows of spines, with 12-16 spines per row, the whole appearing as a wide, closely set band.

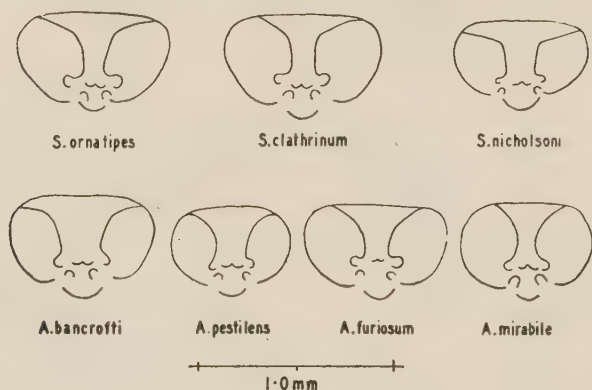


Fig. 9.—Adults. Front view of heads of females.

Pupa

Length 2.5 to 3 mm. Head smooth, cephalic hairs fine, longer than in *S. ornatipes*. Anterior part of thorax smooth, except for a row of small, flat tubercles on each side of the suture line; posterior part of dorsum covered with small tubercles. Thoracic hairs fine, simple, moderately long. Abdominal segments armed similarly to *S. ornatipes*, except that the venter of the fourth segment bears a pair of stout hairs instead of hooks. There are four slender gill filaments arising from a narrow, dark stem on each side (Fig. 7). Each filament tapers to a fine point. The stem and filaments are covered by irregular interlacing rows of minute, black spines, which give them a dark appearance.

Cocoon

Shoe-shaped (Fig. 8), rather coarsely woven, anterior margin produced into a number of interlacing loops, which narrow the opening and give a lattice-like appearance, from which the name is derived (*clathri* - lattice). The gill

filaments, which only protrude a little through the opening in life, are usually bunched together, with the filaments intercrossing.

Female

Length: 2.3 to 2.9 mm.; wing 2.5 mm.

Head: Frons narrow, about one-seventh head width (Fig. 9), brown, with greyish reflections; lanceolate scales down either side, leaving a narrow tomentose strip in middle. Face with grey tomentum, rather densely covered with similar pale scales. Antennae with basal two segments brownish-yellow, extreme base of third brownish-yellow, remainder almost black, with very fine white hairs, form as in Figure 10b. Mouth-parts and palpi blackish.

Thorax: Scutum black, covered for the most part with black lanceolate scales; with a zone of creamy ones along each lateral margin and across front of the scutum, and three somewhat indefinite golden stripes occupying the median and dorsocentral lines; area in front of scutellum rather densely covered with dark golden scales, and a small, dark golden patch anteriorly in each sub-lateral area. Scutellum black, with dark golden scales. Pleurae dark grey, with a conspicuous prealar patch of white, lanceolate scales, and a small tuft of black upper mesepimeral hairs.

Wings: Costa black, R_1 dark brown, remaining veins light brown. The stem vein and other veins making up the triangle at the base of the wing, including the alular area, are almost black, and the membrane in this area appears to be suffused with grey, so that, in the live specimen with the wings held back, there is quite a conspicuous broad black band across the body behind the silvery sheen on the posterior part of the scutum. This contrasts quite strongly with the appearance in *S. ornatipes* and *S. nicholsoni*. Halteres with knob pale lemon-yellow, stem and base dark brown.

Legs: Appear to be entirely dark, except for basal four-fifths of hind metatarsi, which are dull creamy-yellow. Fore legs—coxae covered anteriorly with conspicuous white hairs; femora with dense, white to creamy-yellow scales, extending half way down the femur anteriorly and to the apex posteriorly; tibia with dense, conspicuous, lateral zone of white scales on basal four-fifths; remaining segments entirely dark. Mid legs—basal two-thirds of femora with white to creamy scales anteriorly, remainder dark; a lateral pale zone on basal four-fifths of tibia extending partly round anterior and posterior aspects; metatarsus with lateral patch of white scales on basal half; remaining segments dark. Hind legs—coxae lacking the conspicuous white hairs of other legs; dark apical fourth of femur better defined; knees yellowish; dark apical portion of tibia also better defined; basal four-fifths of metatarsus with pale scales on all sides; second tarsal narrowly pale at extreme base; remaining segments dark. The zones of silvery scales on the tibiae are more extensive, denser and more conspicuous than in *S. nicholsoni*. Hind tibiae distinctly swollen, and somewhat angulated in distal fourth. Calcipala, pedisulcus, and claws as in Figure 11b. As in *S. nicholsoni*,

some specimens show quite conspicuous pale markings on legs underlying the scales described above.

Abdomen: Completely black dorsally, except for the fringe on the first segment, which forms a narrow transverse white line across the base of the abdomen, and a lunulate grey zone at base of second segment. Second to fourth segments and sublateral part of fifth segment covered with black lanceolate scales, remaining segments rather shiny. Lateral margin with conspicuous zones of white hairs on second to seventh segments, most clearly defined on the second, third, and fourth. Venter brown, much darker posteriorly, the colour extending up the lateral aspect of the second segment. Genital fork as in Figure 12b.

Male

Face with ashy tomentum and silvery lanceolate scales. Colouration of antennae similar to female, except that the basal two segments are streaked longitudinally with dark brown. Mouth-parts and palpi black. Scutum black, covered with black and dark bronze scales across the central zone; anterior, lateral and posterior zones covered with dark golden scales, and there are indefinite median and dorsocentral stripes of dark gold; the golden hairs of the lateral zone show almost creamy in certain lights. Scutellum black, with dark gold hairs. Pleurae dark grey, with creamy zone in front of wing-root; prealar patch of scales as in female. Wings and halteres as in female, except that knob of halter is darker yellow. Legs darker than female, and zones of cream or silvery scales more restricted, forming a patch only on basal third of mid and hind tibiae, and being absent from mid metatarsus.

Abdomen black dorsally, except for a conspicuous ashy patch sublaterally on second segment. Most of the dorsum is covered with velvety tomentum, except the sublateral areas of the fifth, sixth, seventh, and eighth segments, which are quite conspicuously shining. Lateral margins with only a trace on the third to fifth segments of the silvery hairs which are so prominent in the female. Venter dark brown, paler at the base. Hypopygium as in Figure 13.

Taxonomic Notes

Holotype female, allotype male, morphotype pupa and larva, from upper Mudgeeraba Ck., near base of Springbrook Mt., 16.iii.47, in collection of Division of Economic Entomology, C.S.I.R., Canberra.

S. clathrinum is a very dark species, which may be taken as representative of the group with prealar scales. The golden lines on the scutum, and bare distal abdominal segments sufficiently distinguish the adults from other members of the group; the pupal gill filaments are much more slender than those of *S. ornatipes* (the only other local species with four filaments), the fenestrated cocoon is quite distinctive, and the larvae are equally distinctive by reason of their compound anal gills.

Biology

Adults have not been taken in the field, and the eggs are unknown. The larvae were restricted to fast moving, clear streams, in which they attached themselves

to stones, grass blades, and occasionally logs. They concentrated where the torrent was most powerful. Pupae occurred, occasionally in quite dense masses, in the same situations.

Distribution

Mudgeeraba Ck., March and May; Little Nerang R. (foot of Springbrook Mt.), March; Mt. Tamborine, March; Albert R., June; Logan R., June (E. N. Marks); Moggill Ck., June; Dawson Ck. (lower slopes of Mt. Glorious), April, South Pine R., April; Brisbane R. (Wivenhoe), May; Gayndah (Barambah Ck.), May, Eidsvold (The Brook, Burnett R.), May.

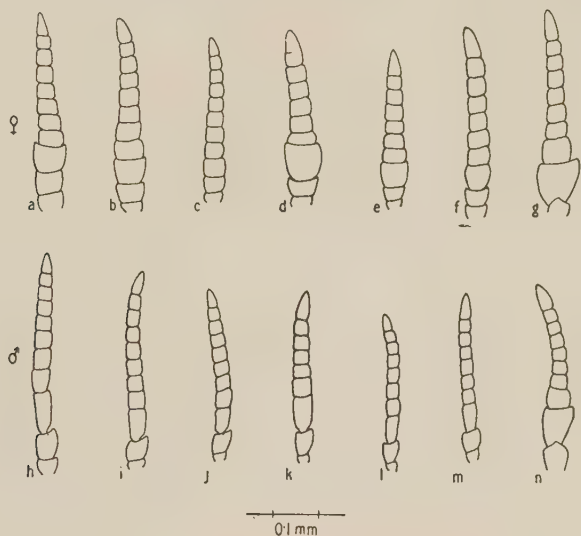


Fig. 10.—Adults. Antennae of females (top row): a. *Simulium ornatipes* Sk.; b. *S. clathrinum* n.sp.; c. *S. nicholsoni* n.sp.; d. *Austrosimulium bancrofti* (Tayl.); e. *A. pestilens* n.sp.; f. *A. furiosum* (Sk.); g. *A. mirabile* n.sp.

Antennae of males (bottom row): h-n in same order as females above.

SIMULIUM NICHOLSONI n.sp.

Larva

Length 4.7 to 5 mm. Body smooth, frequently with a yellow or greenish tint, and faintly mottled with brown or grey markings, head rather pale. Pattern on dorsum of head variable; a frequent arrangement is seen in Figure 3. Submentum with 4 or 5 spines on each side, submental teeth and antenna as in Figure 4c. Gill-spot small, pear-shaped (Fig. 5). Rectal gills consist of three simple digitations. Anal armature as in Figure 6. Two ventral papillae present. Posterior circlet consists of 60 to 70 rows of spines with about 10-12 spines per row.

Pupa

Length 2.5 mm. Head and thorax covered with small flat tubercles, cephalic and thoracic hairs fine, simple, inconspicuous. Abdominal armature essentially

the same as in *S. ornatipes*, except that a few backwardly-directed, flat, sub-basal spines are present on the dorsum of the sixth segment, and that the ventral surface of the fourth segment bears two pairs of simple hooks.

The gill filaments are six in number, and arise from a short, slender stem on each side (Fig. 7). They are narrow, thin-walled tubes, which taper very slightly to a rounded tip. The stem is covered with very minute spines.

Cocoon

Wall-pocket type, finely woven, the anterior margin thickened to form a narrow but definite rim. The sides of the cocoon do not meet anteriorly, and the ventral wall is incomplete, covering only the abdomen of the pupa (Fig. 8).

Female

Length: 2.0 to 2.4 mm.; wing 2.0 to 2.2 mm.

Head: Frons one-quarter head width, with grey pubescence and lanceolate creamy scales (Fig. 9); face similar. Mouth-parts brown; palp with penultimate segment black, terminal segment dark greyish-brown. Antennae with first two and base of third segment brownish-yellow, remainder deep brown, with fine white hairs; form as in Figure 10c.

Thorax: Scutum densely covered with lanceolate scales, predominantly golden in centre, more silvery towards sides; with dorsocentral, rather broad lines of black scales, which converge sharply in anterior quarter and diverge gradually towards scutellum. These lines are often vague and ill-defined. There is an incomplete transverse dark line behind shoulders, and darker markings in posterior part of sublateral areas. Scutellum black, with pale golden hairs on disc and black marginal fringes. Pleurae dark grey, with a conspicuous prealar patch of silvery scales.

Wings: Costa dark brown, remaining veins yellowish-brown. The stem vein and other veins of the wing-root are light brown in colour. Halteres with stem brownish, knob creamy to lemon-yellow in fresh specimens.

Legs: Almost black, with yellowish-brown knees, most conspicuous posteriorly; some suffusion of yellowish-brown underlying the white scales on hind tibiae, and more definitely on basal four-fifths of hind metatarsi. Anterodorsal surfaces of femora, basal two-thirds of tibiae, and basal four-fifths of hind metatarsi densely clothed with silvery lanceolate scales. Fore and mid metatarsi and all distal tarsi black. In some specimens the paler colouration, especially of the hind legs, is more marked and suggests *S. ornatipes*, but the silvery scale patches are distinctive. Calcipala and pedisulcus as in Figure 11c. The base of the claw is humped, but there is no tooth.

Abdomen: Black dorsally, bearing numerous but rather irregularly disposed large, pale, lanceolate scales, which tend to form apical bands on some of the segments; these pale scales are rather dense and conspicuous laterally. First segment yellowish-brown apically, with pale fringe, the lighter colour sometimes suffusing the whole segment. Venter dark brown. Genital fork as in Figure 12c.

Male

Face with silvery tomentum and conspicuous, pale creamy scales. Mouth-parts and palpi brown. Antennae (Fig. 10j) with basal two segments dark brown, somewhat fulvous on inside, remaining segments brown, covered with minute, shining, pale pubescence. Thorax densely covered with almost black scales, mixed with bronzy ones over the greater part of the disc; with patches of gold on each side of the midline anteriorly and in front of scutellum, becoming almost ashy white on anterior, lateral and posterior margins of scutum. Scutellum dark brown, with blackish hairs. Metanotum deep brown. Pleurae pale grey, rather shining; prealar scales conspicuous, silvery. Wings normal. Halteres with stem fuscous, knob lemon-yellow.

Legs with all coxae black, covered with silvery scales anteriorly. Femora black, with some suffusion of brownish-yellow posteriorly, especially on the hind legs, and with rather dense but speckled covering of shining, white scales. Knees brownish-yellow. Tibiae with paler colouration underlying the dense, white scales on the basal half, most conspicuously on the hind legs. Fore and mid metatarsi black; hind with basal four-fifths somewhat indefinitely brownish-yellow beneath the covering of white scales. Remaining tarsal segments entirely dark.

Abdomen velvety black dorsally, brownish-yellow ventrally, with the black restricted by rather shining, grey, sublateral patches on the fifth to the seventh segments (similar to *A. bancrofti*, but not so conspicuous). These patches are sometimes extensive enough to reduce the black areas on 5th and 6th segments to large, median, isolated patches. There is a fringe of golden hairs apically on 1st segment, and rather indefinitely at the sides of subsequent segments. Hypopygium as in Figure 13.

Taxonomic Notes

Holotype female, allotype male, and morphotype pupa and larva, from Brisbane R., Wivenhoe, April 1947, in the collection of Division of Economic Entomology, C.S.I.R., Canberra.

S. nicholsoni is a smaller, more silvery species than *S. clathrinum*, lacks the golden lines on the scutum, and has the distal abdominal segments fully covered with scales. The pupae and cocoons are quite distinctive, while their distinctly yellowish colour usually makes the larvae easily recognizable in the field. The differences between *S. nicholsoni* and *S. faheyi* are noted below.

Biology

This is a wide ranging and abundant species, but we know little about the habits of the adults. Miss Marks has taken two specimens biting at Springbrook, in April, and we obtained a small series, mostly males, by assiduous sweeping of *Melaleuca* spp. in the bed of Barambah Creek, near Kilcoy, and one male on *Melaleuca* in the Callide River near Calliungal. Females have not been observed ovipositing, and the eggs are unknown. In the laboratory, two bred females took human blood fairly readily, but none of the bred adults survived for long, and

circumstances have so far prevented us from following up this initially encouraging observation.

Larvae and pupae occur in the larger, moderately fast streams, sometimes in company with *A. bancrofti*, sometimes with *S. clathrinum*, and sometimes with *S. ornatipes*. Occupying an intermediate position in relation to speed of water, they are found more often in company with another species than alone, though they do occur alone in water that is not fast enough for *A. bancrofti* and not clear enough for *S. ornatipes*. They seem to be equally at home in inland, fairly muddy waters as in clear, coastal streams. They will attach to any hard, clean surface, though they are perhaps most frequent on twigs, broken branches, and dead leaves caught on obstruction within about 6 inches of the surface in places where the water is flowing freely.

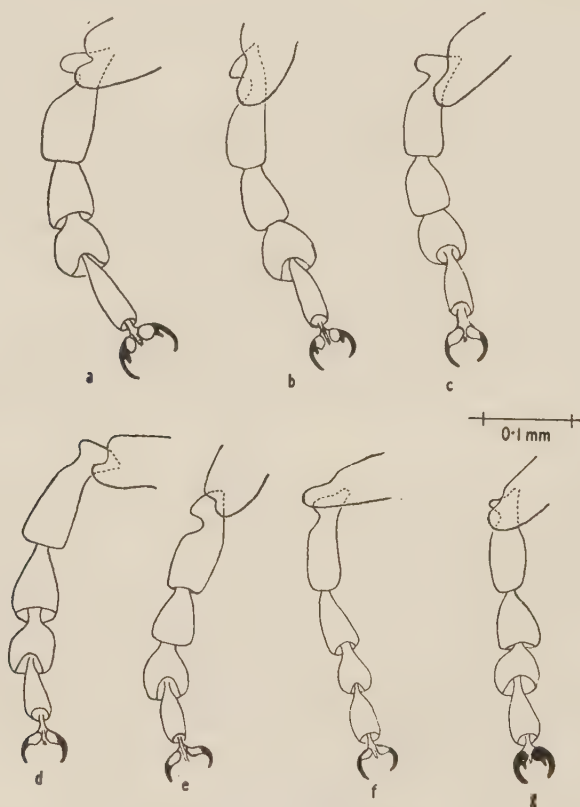


Fig. 11.—Adults. Tarsal segments of females of: a. *Simulium ornatipes* Sk.; b. *S. clathrinum* n.sp.; c. *S. nicholsoni* n.sp.; d. *Austrosimulium bancrofti* (Tayl.); e. *A. pestilens* n.sp.; f. *A. furiosum* (Sk.); g. *A. mirabile* n.sp.

In the laboratory, the pupal stage lasted 2 to 3 days in summer. Flies emerged normally from pupae which were removed from water and allowed to become almost completely dry. In one instance, we even got emergence from pupae

formed on the moist vegetation by larvae which were clinging to it when it was taken from the stream. There seems little doubt that pupae left exposed by falling water levels would complete their development normally in the field.

Distribution

Brisbane district—Brookfield, March; South Coast—Mudgeeraba Ck., March; Albert R., June; Springbrook, April (E. N. Marks); Logan R., June; Dalby district (Back Ck.), April; Brisbane R. (Wivenhoe and Murrumba Crossing), April, May; Nanango, April; Goomeri (Barambah Ck. and Boonara Ck.), April and May; Gayndah (Burnett R. and Reed's Ck.), April and May; Eidsvold (Burnett R. and many creeks in district), May; Dawson R. (Theodore and Joe's Ck.), April; Dee R., Rannes, April; Don and Callide Rs., Calliungal, April and May; Mackenzie R., June (J. L. Wilson).

SIMULIUM FAHEYI Taylor

Simulium faheyi Taylor, 1927, p. 71.

Female

Head: Frons dark grey, with creamy scales; tapering from about one-third head width at vertex to about one-sixth above antennae. Palpi and mouth-parts dark brown. Antennae with basal two segments light creamy-brown, third bright brown, remainder dark brown.

Thorax: Scutum deep brownish-black, covered with bright, pale golden scales, which shine a little silvery on the shoulders; indefinite dorsocentral bronzy stripes, similarly arranged to the darker ones of *S. nicholsoni*. Pleura varying from brown to dark grey, with an ashy sheen extending down below anterior spiracle; prealar scales golden, conspicuous.

Wings: Veins brown and macrotrichia black. There is some darkening of the concentration of veins at the wing-root, and the membrane in this area is distinctly clouded. Halteres light brown throughout, with very little darkening towards base of stem.

Legs: Normal, except for knees, basal half of hind tibiae, and basal five-sixths of hind metatarsi, all of which are creamy in colour. Pale scales on legs golden, except on paler areas mentioned, where they also show paler reflections. Hind tibiae with smoothly curved contour. Calcipala normal (i.e. a little longer than wide), covering about half the second tarsal segment; pedisulcus a well-marked notch. Claws with a minute tooth forming little more than a pointed apex to the basal thickening.

Abdomen: Somewhat twisted and shrunken. First segment brown, with bright golden-brown fringe; remaining segments deep brown, with pale golden scales across the disc of the fifth and subsequent segments. Laterally, the patches of bright, pale golden scales occur on the third and subsequent segments, and are most conspicuous on the third. Venter hidden.

Taxonomic Notes

The above notes were made during a rather brief examination of the type in the School of Public Health and Tropical Medicine. No parts were mounted for detailed study. It may be mentioned that this specimen is labelled "Allotype," although it is unique, and that it is a female, although Taylor (1927) was uncertain of the sex. The type of *A. bancrofti* (a female) is also labelled "Allotype," and it appears that, at one stage, Taylor used "Holotype" for male and "Allotype" for female types, irrespective of whether or not he had the other sex.

S. faheyi is close to *S. nicholsoni*, but we believe that it is distinct by reason of its general rich brown and golden, rather than black and silvery to cream, adornment, golden rather than creamy prealar scales, and presence of a tooth (though minute) on the claws. There must necessarily remain some doubt, until the early stages are discovered.

Distribution

Innisfail, North Queensland (F. H. Taylor), a female taken while sweeping with a net along a creek bank. We also, with some hesitation, place here a female taken by one of us at Lawn Hill, west of Burketown, in May 1931.

VII. THE GENUS *AUSTROSIMULIUM* TONNOIR*AUSTROSIMULIUM BANCROFTI* (Taylor)

Simulium bancrofti Taylor, 1918, p. 168; 1927, p. 70 (part).

Austrosimulium bancrofti (Taylor), Tonnoir, 1925, p. 241 (part); Drummond, 1931, p. 8.

Larva

Length about 5 mm. Body creamy, with darker mottling. Head usually fairly heavily pigmented. Pattern on the dorsum of head very variable, a common arrangement being shown in Figure 3. Submental teeth reduced in number, arranged as in Figure 4d; antennae as in Figure 4d (left). Gill-spot elongate, club-shaped, as in Figure 5. Rectal gills three simple digitations. Anal armature as in Figure 6. Ventral papillae absent. The posterior circlet consists of over 90 closely-set rows of spines, with 20 to 30 spines per row.

Pupa

Length 2.5 mm. Head covered with small, irregularly-disposed tubercles. There is a row of tubercles on each side of the suture line of the thorax, and laterally the thorax is covered with tubercles arranged in tiny rosettes, an area on each side of the midline being left bare. Cephalic and thoracic hairs fine, simple. The hooks on the dorsum of segments three and four of the abdomen are widely spaced; four pairs of similar hooks also occur on the fifth, sixth, and seventh segments, and two pairs on the eighth segment. There are no dorsal, backwardly-directed, flat spines, nor ventral hooks, such as are present in *Simulium*.

The respiratory organ on each side consists of a broad, flat, spatulate, basal horn, covered with small spines, which are particularly conspicuous towards the tip. Very numerous, fine filaments originate from the whole of the outer surface

of the horn, except for the distal fourth (Fig. 7); they are about the length of the horn itself or a little shorter, and are transversely banded.

Cocoon

Shoe-shaped (Fig. 8), fairly closely woven, with a conspicuous anterior collar, but without a thickened rim. The ventral wall is incomplete posteriorly, the whole of the abdomen usually resting on the substrate.

Female

Length: Variable, 2 to 3 mm.; wing 2 to 2.5 mm.

Head: Frons about one-third head width (Fig. 9), covered with grey tomentum, with pale creamy hairs showing dull yellowish in some lights; occiput similar. Face with ashy white tomentum and creamy-white hairs. Palpi and mouth-parts dark brown. Antennae 9-segmented (Fig. 10d); basal segments creamy-yellow, remainder brownish-black. The third segment is nearly twice as long as the second and distinctly expanded dorsoventrally, the ninth is large, conical, about twice as long as the eighth, and often with a notch or groove near its middle.

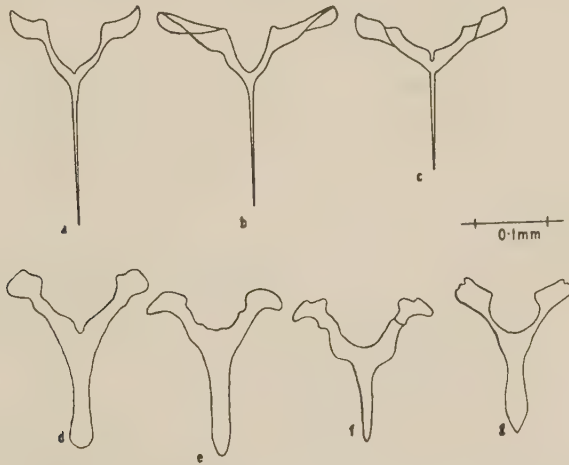


Fig. 12.—Adults. Genital forks of females. Order as in Figure 13.

Thorax: Scutum covered with dark grey tomentum and fairly dense short hairs, which are dull golden over most of the disc, but show silvery on the lateral and anterior margins. Area in front of scutellum with longer, rather creamy hairs. Scutellum black, with similar hairs, and a line of long, darker hairs along apex. Metanotum black, with some ashy reflections. Pleurae covered with ashy tomentum.

Wings: Veins pale fawn, hairs on main veins black. Halteres with basal part of stem dark, distal part of stem and knob pale creamy-yellow.

Legs: Dark grey, with considerable pale fulvous suffusion and silvery hairs, the fulvous colour being distributed as follows: Fore leg—knee, tip of tibia

ventrally; mid leg—base of femur, knee, tip of tibia, basal half of metatarsus, base of second tarsal and trace on base of third tarsal; hind leg—base of femur, knee, portion of ventral aspect of tibia, narrowly on tibio-metatarsal joint, narrowly on basal part of second tarsal segment. Calcipala about half width of metatarsus and about as long as wide (Fig. 11d). The claws are not toothed.

Abdomen: Predominantly ashy, forming a conspicuous pattern, the velvety black ground colour being reduced to a lateral zone on second, third, and fourth segments, which is broken into two dark stripes on fifth, sixth, and seventh segments. These dark markings on each side are joined by narrow basal dark bands on all the segments. There are also small ventrolateral basal brown patches on fifth, sixth, and seventh segments, and the venter is light fulvous, which colour extends more widely beneath the basal four segments than the ensuing three. Genital fork as in Figure 12d.

Male

Antennae (Fig. 10k) with all segments dark, except for traces of yellow on the first and second; third segment not as broad as in female, but broader than is usual in males; ninth olive-shaped, nearly twice as long as eighth. Mouth-parts and palpi dark brown, almost black.

Scutum covered with jet black tomentum, mixed with dark golden and black hairs, the gold being less dense than in female. Shoulder and lateral margin to wing-root with ashy tomentum and paler hairs. Scutellum jet black, with long black hairs. Metanotum black, with some ashy reflections. Pleurae covered with grey tomentum. Wings as in female. Halteres with stem dark brown, knob brilliant lemon-yellow.

Legs dark grey, with diffuse pale fulvous markings as follows: fore leg—on posterior aspect of femur, knee, inner aspect of tibia; mid leg—similar, but also includes basal part of metatarsus and extreme base of second tarsal segments; hind leg—more extensively pallid on anterior face of femur and tibia, but metatarsus all dark, base of second tarsal segment pale.

Abdomen jet velvety black dorsally, and brownish-fulvous on venter, with a conspicuous patch of brilliant ashy tomentum laterally on fifth and sixth segments; this involves also the apical quarter of the fourth segment, and sometimes part or whole of the seventh. Fringe of first segment dark dorsally, bright silvery and conspicuous laterally. Hypopygium as in Figure 13.

Taxonomic Notes

There has been much confusion about the identity of *A. bancrofti*, Taylor maintaining that it had nine-segmented antennae, and Tonnoir that it normally had ten. The type series consists of nine females, all bearing a printed label "Eidsvold, Queensland, Dr. T. L. Bancroft" evidently affixed by Taylor. The type specimen is labelled "Allotype," and has nine-segmented antennae, with a large, broad third segment as illustrated by Taylor (1927) in his Figure 2. A specimen labelled "Paratype" has ten-segmented antennae of the form shown in Taylor's Figure 1. Of the others, one has nine-segmented antennae, and six have ten-segmented

antennae. Thus, the type series comprised two *A. bancrofti* and seven *A. pestilens*! Tonnóir also had both forms before him in 1925, but later (MS.) correctly placed this species and prepared notes on its life-history. Drummond (1931) also recognized it correctly, and, incidentally, designated an allotype male, which he lodged in the collection of the Division of Economic Entomology, C.S.I.R., Canberra.

Adults of *A. bancrofti* can be immediately distinguished from all other species of the genus, except *A. pestilens*, by the abdominal markings in both sexes. From *A. pestilens*, it is only separable by the antennal characters, although the larvae and pupae are quite distinct.

Biology

The adults of *A. bancrofti* in Queensland are obscure, unobtrusive creatures. The few specimens captured by Bancroft (and ourselves in earlier visits to Eidsvold) were taken biting either man or horse. During a recent visit to the district, we were not bitten once by *A. bancrofti*, though we did collect a few individuals by sweeping *Melaleuca* in the stream-beds. The situation was very remarkable and instructive. Breeding of *A. pestilens* had ceased, but there were countless thousands of larvae and pupae of *A. bancrofti* in the river, and adults must have been emerging in very large numbers. Every individual of the many we bred out had nine-segmented antennae, and yet every one of the few adults that bit us had ten-segmented antennae! They represented the last survivors of the *A. pestilens* wave we were too late to see in that area. Further south, *A. bancrofti* seems to be more interested in human blood, and there is quite a series in the collection of the Division of Economic Entomology bearing labels that they had been taken biting man in the Australian Capital Territory. Drummond (1931) records it as biting fiercely in south-western Australia.

The early stages were always found in swift, strongly flowing water (about 4 feet per second), sometimes clear, but usually opalescent, and often quite muddy, though neither as swift nor as muddy as was favoured by *A. pestilens*. They attached themselves to any clean substrate, often on smooth, water-worn stones, but especially on broken limbs and logs caught in the current (Plate 2, Fig. 1). They occurred at any depth from the surface down to 15 inches and perhaps further, often alone, but not infrequently associated with either *A. pestilens* or *S. nicholsoni*, depending on the speed and muddiness of the water.

The larvae of *A. bancrofti* showed one habit which we have not seen in any other species except *A. pestilens* (and once in *A. clathrinum* in particularly fast water). They do not spread out in evenly spaced array, but crowd together in dense masses, more like maggots in a carcass than *Simulium* larvae in a stream. In favourable places one can almost pick them up by the handful.

When about to pupate, the larvae of *A. bancrofti* tend to seek out crevices and crannies a little sheltered from the direct force of the water. They usually pupate in little lines and groups, with each pupa having at least some direct hold on the substrate, but sometimes crowd together in a manner reminiscent of *A. pestilens*.

The pupal stage lasted two to three days. Even freshly formed pupae emerged normally when removed from the water.

Distribution

South Coast—Mudgeeraba Ck., March and May, Albert R., June; Dalby district (Back Ck.), April; Chinchilla (Charley Ck.), April; Goondiwindi (McIntyre R.), August, J. L. Wassel; Roma district (Muckadilla Ck.), March, R. A. J. Meyers; Brisbane R. (Wivenhoe and Murrumba Crossing), April and May; Goomeri district (Boonara Ck. and Upper Barambah Ck.), April and May; Gayndah district (Lower Barambah Ck.), April and May; Eidsvold district, Burnett R., Lochaber Ck. (W. J. Roulston), The Brook, Jacky Small's Ck., April and May; Dawson R., near Theodore, April; Dee R., Rannes, April; Don. R. and Callide R., Calliungal, April and May; Mackenzie R., April and June (J. L. Wilson).

Also recorded from A.C.T. (Tonnoir, MS.) and south-western Australia (Drummond 1931). The distribution of *A. bancrofti* is probably much more extensive than our records indicate, but will not be fully known until collections are made of larvae and pupae rather than adults.

AUSTROSIMULIUM PESTILENS n.sp.

Simulium bancrofti, part, Taylor, 1927, p. 70.

Austrosimulium bancrofti, part, Tonnoir, 1925, p. 241.

Larva

Length 4.5 to 5 mm. Body pale, with the usual irregular mottling. Head very pale, appendages lightly pigmented. Head pattern very often inconspicuous, a pattern sometimes observed being shown in Figure 3. Submentum and antenna as in Figure 4e. Gill-spot small, oval, as in Figure 5. Rectal gills three simple digitations. Anal armature slender, as in Figure 6. Posterior circle consists of 70 to 80 rows of spines, 10 to 12 spines per row. Ventral papillae absent.

Pupa

Length 2.2 to 2.6 mm. Head and thorax covered with small tubercles. Cephalic and thoracic hairs relatively well developed, the most posterior of the dorsocentral row on the thorax being quite stout and forwardly curved. Abdominal armature resembles *S. ornatipes* in the disposition of the hooks on the dorsal surface of the third and fourth segments, but it resembles *A. bancrofti* in the absence of flat, backwardly-directed teeth and ventral hooks. In place of the dorsal hooks of *A. bancrofti* on the fifth to the ninth segments, *A. pestilens* has stout, curly hairs. The terminal pair of hooks is well developed.

The respiratory organ on each side consists of a slender, pointed basal horn, from which numerous fine filaments arise (Fig. 7). The surface of the horn is irregular, and the filaments, which are finely banded, spring from all parts of it. They are a little longer than the horn itself.

Cocoon

Varies a good deal in shape owing to the habit of building them one on top of another. Sometimes it is definitely shoe-shaped, with a well-developed collar, as in *A. bancrofti* but shorter; while in others the sides merely meet in the midline in front (Fig. 8). Fairly closely woven, with a slight rim around the opening, but no central dorsal projection.

Female

Length: Body 2 to 2.5 mm.; wing 1.8 to 2 mm.

Head: Small; frons (Fig. 9) about one-fourth of head width, i.e. a little narrower than in *A. bancrofti*, but variable. Antennae ten-segmented (Fig. 10e); third segment a little larger than second, tenth conical, shorter than in *A. bancrofti*; all segments dark.

Thorax: Similar to *A. bancrofti*, but the ground colour darker, as are the pleurae and legs. Calcipala and pedisulcus as in Figure 11e; claws not toothed. Wings as in *A. bancrofti*.

Abdomen: With segments brown, marginal hairs of first less conspicuous than in *A. bancrofti*, but the pattern is similar. Central ashy stripe hardly visible on second segment, indefinite on third and fourth, becoming more conspicuous on fifth, and expanding on sixth and seventh. Lateral margins with apical, lunulate, ashy patches tapering to a point medially. Genital fork as in Figure 12e.

Male

Antennae entirely dark; third segment half as long again as second but relatively narrower, tenth conical (Fig. 10b). Thorax black, somewhat shiny, with sparse pale golden hairs. Pleurae dark. Legs dark, with silvery hairs externally on femora, tibiae, and metatarsi.

Abdomen black; sides brownish, with the brown extending along apical edge of segments to form a narrow band dorsally. Venter fawn coloured. There is an indication of ashy reflections on some of the abdominal segments laterally, though usually not as conspicuous as in *A. bancrofti*. Hypopygium as in Figure 13.

Taxonomic Notes

Holotype female, allotype male, and morphotype pupa and larva, from Charley's Creek, Chinchilla, April 1947, are in the collection of the Division of Economic Entomology, C.S.I.R., Canberra.

A. pestilens is nearly related to *A. bancrofti*, from which the adults are only to be separated with certainty by possessing ten-segmented antennae. We have examined very many bred specimens of both species, and have not found the number of segments to vary in either, although they are often difficult to count in dried specimens. Moreover, all adults we collected biting in various parts of Queensland during 1947, and all the extensive series submitted by stock inspectors as worrying stock, had ten-segmented antennae. In the female, the form and size of the third antennal segment is rather characteristic (cf. Figs. 10d

and 10e), and the head of *A. pestilens* is distinctly smaller than that of *A. bancrofti* (Fig. 9).

The breathing horn of the pupa is quite different in the two species (Fig. 7), and the cocoons are usually sufficiently distinct to be recognized with a hand lens. Full grown larvae can also be readily distinguished with a hand lens by the form of the pupal gill-spot, which is particularly distinctive in *A. bancrofti* (Fig. 5). Other distinguishing larval characters are the pallor of the head in *A. pestilens*, and its delicate anal armature with more divergent anterior limbs.

Biology

This is the pest Simuliid of inland Queensland. Its original hosts were probably kangaroos and wallabies (*Macropus* spp.), and we have been informed by stock-owners that these marsupials sometimes die from the severity of the worry during a bad sandfly wave. Now they attack man, cattle, horses, sheep, and dogs with equal avidity. Cattle are driven from the waters, and mill slowly round in a mob all day, stirring up a cloud of dust, which gives them some protection. It is said that there is never any need to muster during a sandfly wave, for all the cattle in the paddock will be found in one compact mob. One grazier told us that he had seen a single mob of 7,000 head put together by sandflies in the Dawson River country. Horses crowd into fires for protection, and often get burnt. Sheep mill round, stirring up dust in the same way as cattle. Calves and lambs become separated from their mothers, and this is considered to be the main cause of the deaths that occur. Roberts (1940) has noted that the eyes and nostrils of young lambs may be blocked, and that the flies are sometimes inhaled, which may also be a cause of death.

Adults of *A. pestilens* are closely associated with tea-trees (especially *Melaleuca branchiata*). They shelter in the foliage after they emerge, and return to the trees to digest their blood meal. Dense clouds of hungry females arise from the bushes as one moves through them, and hundreds of flies may be collected with a single sweep of the net. Females are more numerous than males in such swept material, and include unengorged and recently engorged individuals, and specimens showing partial digestion of a previous blood meal. We did not see adults very far from the streams in which they bred, but apparently they follow the stock out, and graziers have recorded them up to ten and twelve miles from the rivers.

A. pestilens would be one of the most dangerous pests of stock in this country, were it not for the fact that its season of activity is extremely short. The fly wave follows flooding in the streams with great regularity about ten days after the waters come down, and lasts only about ten to fourteen days. There is no more worry, unless further flooding occurs, when secondary or tertiary waves may follow. At these times, the whole country is a sea of mud, which gave rise to the widely held opinion that the flies breed in mud. The brevity of the season is quite remarkable. Thus, flies were present in thousands, but larvae and pupae

in reduced numbers, in the Don River at Calliungal on April 13. By April 29 larvae and pupae had disappeared (empty pupal shells in abundance above the now lower water level), males also disappeared, and only a very few engorged females were taken by thorough sweeping of the bushes.

These observations can be related to the restricted habitats of the early stages. Larvae and pupae were only found when the streams were torrential (water speed in excess of 4 feet per second), turbulent, and muddy. Then they were extraordinarily numerous, larvae crowding in masses like *A. bancrofti*, and the pupae too being piled on top of one another, as if there were not enough space to accommodate them all. They would attach to sticks, logs, stumps, and dead leaves caught in a submerged limb, but by far the most favoured situation was on submerged branches and fronds of living *Melaleuca branchiata*. Later, when the level fell and the speed of the water decreased, larvae and living pupae disappeared; we could find no indication of persistent breeding even on a reduced scale, only the masses of empty pupal shells in their cocoons, now feet above the water, to mark the intense proliferation that had occurred (Plate 2, Fig. 2).

Collecting the early stages is a muddy, amphibious operation, so it is perhaps not surprising that they have not been described before, but it is surprising that only Mr. John Mann recognized them and the zone of dried cocoons left after the fly wave disappeared.

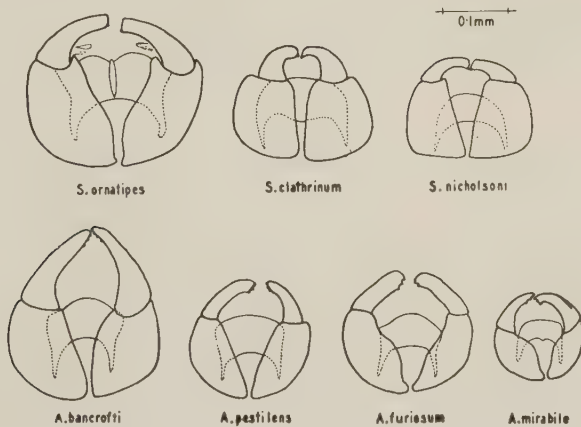


Fig. 13.—Adults. Hypopygia of males.

We have no doubt left in our minds that *A. pestilens* (and also *A. bancrofti*, *S. nicholsoni*, and *S. ornatipes* in the western parts of their range) has a resistant stage in which it lies dormant between waves. What that stage is is not so clear. Time relations of the outbreaks strongly suggest that it is the egg. If development were more or less complete before the egg dried (as in some mosquitoes), then about a week for development of the larvae and two days for the pupae would fit the picture very well. We have, however, not yet found the eggs, and

have no information about the duration of any stage except the pupal, so the sequence is purely conjecture. Much dried material was collected in the field, but so far no living stages have been demonstrated in it.

Control

This is not the place to discuss control at any length, especially as we have no experimental evidence to offer. At present, little is done other than to build smudge fires (Roberts 1940) to protect men and horses. These are remarkably effective within their limits, for the flies are extremely sensitive to acrid smoke. One could, for example, work quite comfortably amongst the tea-tree at the river edge, protected by three or four "incense-pots" of burning cow dung, when it would have been quite impossible even to remain there without the smoke.

DDT gives considerable promise for the control of Simuliidae. Fairchild and Barreda (1945) in South America, and Garnham and McMahon (1947) in Kenya, had considerable success by adding DDT to the stream in very low concentrations. This might present practical difficulties in Queensland, for the time to treat would be during the week following flooding, and much of the country is then impassable. Spraying from aircraft delivering a single, long, continuous swathe following the central line of the most turbulent water might be the answer to this difficulty. A third possibility is to use the dips charged with DDT for cattle-tick control to turn the cattle into poison baits for the flies. This also presents the practical difficulty that it may not be possible to handle the stock when dipping would be most effective. Nevertheless, it is felt that all three methods are worth a trial to determine their effectiveness and limitations.

Distribution

Widely distributed in the southern half of Queensland, west of the Dividing Range, and extending as far as the Cooper's Creek basin. East of the Divide, it has only been found in great numbers in the Dawson River area (where it is so abundant that it is widely known as the "Dawson River sandfly") and the headwaters of some tributaries of the Burnett River. We have seen specimens (adults or early stages) from the following localities: Dirranbandi (January), Cunnamulla (March), Wooroorooka (April), Chinchilla (March, April), Roma (April), Charleville (March), Adavale (March), Windorah (March), Boondoomba (March), Cadarga (April), Goomeri (April), Gayndah (April), Eidsvold (May), Theodore (April), Calliungal (April).

AUSTROSIMULIUM FURIOSUM (Skuse)

Simulium furiosum Skuse, 1888, p. 1362.

Austrosimulium furiosum (Skuse), Tonnoir, 1925, p. 239.

Larva

Length about 5.5 mm. Body greyish, with the usual irregular mottling. Pattern on the dorsum of the head very variable, a common arrangement is shown in Figure 3. Submentum and antenna as in Figure 4f. Gill-spot large, S-shaped,

as in Figure 5. Rectal gills three simple digitations. Anal armature as in Figure 6. Posterior circlet consists of 70-80 rows of spines, 10-12 spines per row. Ventral papillae present.

Pupa

Length 2.3 to 2.5 mm. Head and thorax covered with small tubercles. Cephalic and thoracic hairs well developed, the most posterior dorsocentral thoracic pair being stout and forwardly curved. Abdominal armature similar to that of *A. pestilens*.

Respiratory organ on each side consists of a slender stem, from the summit of which about 23 long, fine filaments arise (Fig. 7). The stem is beset with fine spines, and the filaments have bands of minute spines giving them a segmented appearance. Some specimens have filaments about three-fifths the length of the usual type.

Cocoon

Wall-pocket type (Fig. 8), with a low collar formed by the sides meeting in front; coarsely woven, with a definite rim, but no central dorsal projection. Ventral wall absent.

Female

Length: 2 to 2.4 mm.; wing 2.2 mm.

Head: Frons about one-sixth of head width (Fig. 9), dark greyish-brown, with short creamy hairs; face similar. Antennae with all segments dark brown; third to tip darker than first two and covered with short, fine, creamy hairs (Fig. 10f). Palpi and mouth-parts brown. Basal segment of palp darker than the rest.

Thorax: Scutum brownish-black, fairly evenly covered with creamy-gold hairs which do not show much contrasting lustre on sides. Scutellum dark brown, with some small pale gold hairs and dark apical ones; postnotum brown. Pleurae dark brown, with three patches of ashy tomentum, one behind anterior spiracle, one below wing-root, and one just behind wing-root.

Wings: Costa and stem vein dark brown, remaining veins paler brown. Halteres with dark stem and creamy knob.

Legs: Dark brown, suffused with a dull fawn hue, especially on femora; distal sixth of the femora and tibiae very dark, almost black; knees yellowish. Calcipala and pedisulcus as in Figure 11f. Claws not toothed.

Abdomen: First segment greyish-brown, with cream fringe; remaining segments all black, without ashy markings. Laterally the colour is rather a greyish black, but centrally each segment is deep velvety black as described by Skuse. Fifth and subsequent segments with pale creamy hairs, which tend to form an indefinite apical fringe; venter brown. Genital fork as in Figure 12f.

Male

Darker than female. Antennae (Fig. 10m) entirely dark; mouth-parts black. Scutum jet black, with fairly uniform covering of dark golden hairs. Scutellum

black, with a few pale hairs and a black marginal fringe; postnotum black, with ashy reflections. Pleurae dark grey, with ashy suffusion running downwards from below the anterior spiracle. Wing veins and knob of halter darker than female. Legs similar to female, but rather darker. Dorsum and sides of abdomen velvety black, without pale hairs except on first segment, where the fringe is greyish-brown but fairly conspicuous nevertheless; venter grey. Hypopygium as in Figure 13.

Taxonomic Notes

Our Queensland specimens agree very well with a series of larvae, pupae, and bred adults obtained by D. Mackerras at Gosford, New South Wales, Skuse's type locality. The females have also been compared with Skuse's original type series by Mr. R. H. Wharton, who informs us that he could discover no significant differences. Unless other larvae and pupae producing identical adults turn up at Gosford (a not impossible contingency), the identity of Skuse's species may be taken as established.

A. furiosum is easy to distinguish in Queensland, for it is the only known representative of the southern group of species with an entirely dark abdomen in both sexes. Larvae and pupae were collected usually in clear, fast-running water, but in one of the Queensland localities they were present in a mere trickle. The latter probably represented the last residue of breeding in a stream which had previously been more vigorous. Adults have not been seen in nature.

Distribution

Coombabah Ck., near Southport, March; Logan R., near foot of Mt. Barney, June (E. N. Marks); Noosa, September (E. N. Marks); Dalby district, Back Ck., April.

AUSTROSIMULIUM MIRABILE n.sp.

Larva

Length about 4.5 mm. Body creamy, with the usual dark mottling. Head usually heavily pigmented, pattern variable, a common arrangement shown in Figure 3. Submentum and antenna as in Figure 4g. Gill-spot conspicuous by reason of the jet-black basal respiratory horn, elongate oval, with truncated upper end (Fig. 5). Rectal gills consist of three simple digitations. Anal armature includes a conspicuous chitinous rod which extends around the body just anterior to the posterior circlet (Fig. 6). Posterior circlet consists of about 80 rows of spines, 12-15 spines per row. Ventral papillae present.

Pupa

Length 2.2 mm. Head and thorax covered with small tubercles. Cephalic and thoracic hairs well developed, particularly the dorsocentral thoracic hairs, the two posterior pairs being stout and forwardly curved. Abdominal armature consists of the usual four pairs of strong hooks dorsally on segments three and four, and a series of ventral hooks similar to those of *S. ornatipes*.

The respiratory organ on each side consists of a dense, black, spiny, pointed horn, from which numerous fine filaments arise (Fig. 7). The surface of the horn is covered with longitudinal spiny ridges, the filaments springing from the furrows between them. The filaments are finely ringed, and are about as long as the horn itself.

Cocoon

Wall-pocket type (Fig. 8), rather coarsely woven, with a definite anterior rim and a short central dorsal projection. The ventral wall is absent, and the sides do not meet anteriorly below the head. The cocoon is usually very much spread out laterally, so that the outline is nearly circular, and only the central part is raised up sufficiently to accommodate the body of the pupa.

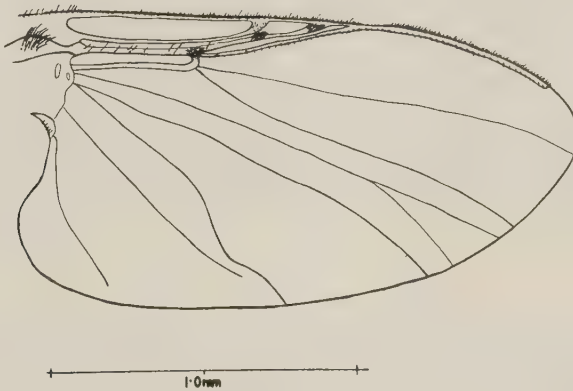


Fig. 14.—Wing of *Austrosimulium mirabile* n.sp., female.

Female

Length: 2.25 mm.; wing 2.25 mm.

Head: Frons narrow (Fig. 9); frons and face silvery; occiput black, with golden hairs. Antennae very long, 10-segmented; first segment large, second very large, more than twice as long as first, third about half as long as second, narrower (Fig. 10g). First three segments black, fourth black at extreme base, remainder of segment orange; fifth and sixth entirely orange; seventh to tenth black. Proboscis dark brown to blackish; palpi dark, except terminal segment, which is pale.

Thorax: Black, with evenly scattered, fine, golden hairs on disc, and a greyish area in front of scutellum. Scutellum black. Pleurae black, with a conspicuous ashy patch of tomentum below edge of scutum and behind pronotal lobes, which bear a silvery pubescence.

Wings: With three conspicuous black spots on R_1 , one opposite the origin of R_s , second opposite the tip of Sc and including it, and third spot at the tip of R_1 . The macrochaetae on R_1 are concentrated in relation to the spots, and there is

also a patch of thick hairs on base of stem vein (Fig. 14). Halteres with stem dark brown, knob pale creamy-yellow.

Legs: Black; femora and tibiae black, knees narrowly yellowish, tarsi dark brown. Claws strongly toothed. Calcipala very large, three-fourths width of segment and covering the pedisulcus (Fig. 11g).

Abdomen: First visible segment brown, with pale edge and long golden fringe laterally. Second dark brown, with an oblong ashy spot on either side of midline and a velvety black patch in centre. Third and fourth entirely velvety black. Fifth with ashy, T-shaped area in centre; base and arms of T marked with brilliant white scales. Sixth similar, but cross arms much wider. Seventh similar, but pale area expanded so that the black portion is reduced to a triangular patch on each side of midline. Eighth segment ashy, with darker suffusion on each side of midline and silvery scales laterally. Ninth darker, with silvery tip. Genital fork as in Figure 12g.

Male

Head wider than thorax; face black, with large, central, shining patch of ashy tomentum. Mouth-parts and basal palpal segments brownish-black, terminal segment creamy-brown. Antennae (Fig. 10n) with first two segments relatively large, as in female, second markedly expanded dorsoventrally; segments one to three brownish-black, four to six orange-yellow, remainder brown, with some orange suffusion and pale hairs more conspicuous towards the tip.

Thorax as in female, but the golden hair is more dense, and the ashy area in front of scutellum is much larger, more conspicuous, and indistinctly divided in the middle by a median patch of long, golden hairs, which extend backwards over base of scutellum. Pleurae and legs as in female.

Abdomen with fringe hairs of first segment much darker than in female. Second segment velvety black in centre, brownish laterally, with conspicuous ashy tomentose bands along anterior and posterior margins. Third and fourth segments all black (no brownish areas differentiated). Fifth segment black, with ashy tomentose patch laterally, and small bright ashy median spot apically. Sixth and seventh black, with larger ashy median apical spots. Eighth similar, but with ashy distal margin in addition to the spot. Ninth brown. Pale median areas of abdomen have patches of silvery scales as in female, but they are smaller. Hypopygium as in Figure 13.

Taxonomic Notes

Holotype female, allotype male, and morphotype pupa and larva, from Dawson Ck., slopes of Mt. Glorious, April, in collection of the Division of Economic Entomology, C.S.I.R., Canberra.

This is a remarkably distinctive species, by the form and colour of the antennae, the spotted wings, and abdominal markings. Pupa and larva are more normal, but still well differentiated from other species. It is only known from the type series, which was collected on dead leaves caught in rocky crevices in moderately fast, clear water.



Fig. 1



Fig. 2



Fig. 1



Fig. 2

MACKERRAS and MACKERRAS.—SIMULIIDAE (DIPTERA) FROM QUEENSLAND

VIII. ACKNOWLEDGMENTS

The work described in this paper was carried out as part of the research programme of the Division of Economic Entomology, C.S.I.R.

Our thanks are due to Dr. F. H. S. Roberts, who first showed us a *Simulium* breeding ground (it proved to be *S. ornatipes*) near Brisbane; to Mr. J. Mann, who guided us to a breeding ground of the pest species near Chinchilla; to Miss E. N. Marks for the first specimens of *Cnephia*; to Mr. K. J. Clinton for information about Taylor's types, and for permitting us to examine them; to Mr. R. H. Wharton for a careful comparison of our material with Skuse's type series of *A. furiosum* (Sk.); and to Mr. D. Mackerras for the "bubbler" and for useful collections, especially for a good series of *A. furiosum* from Skuse's type locality.

Most of the material studied was collected by the authors in streams in Southern Queensland, but we are indebted to Miss E. N. Marks and to Messrs. J. Mann, R. A. J. Meyers, and J. L. Wilson for additional material. Through the good offices of Dr. F. H. S. Roberts we were also able to examine large numbers of adults of the pest species collected by Stock Inspectors in many parts of Southern and Western Queensland.

We are grateful, finally, to many friends in the country for accommodation and help during the investigations.

IX. REFERENCES

- DRUMMOND, F. H. N. (1931).—West Australian Simuliidae. *J. Roy. Soc. W. Aust.* 18: 1-12.
- EDWARDS, F. W. (1931).—Diptera of Patagonia and South Chile. Part II, Fasc. 1. Simuliidae. *Brit. Mus. (Nat. Hist.)* 1931: 121-54.
- EDWARDS, F. W. (1934).—Deutsche Limnologische Sunda-Expedition. The Simuliidae (Diptera) of Java and Sumatra. *Arch. Hydrobiol.*, Suppl. 13: 92-138.
- FAIRCHILD, G. B., and BARREDA, E. A. (1945).—DDT as a larvicide against *Simulium*. *J. Econ. Ent.* 38: 694-9.
- GARNHAM, P. C. C., and MCMAHON, J. P. (1947).—The eradication of *Simulium neavei* Roubaud from an onchocerciasis area in Kenya Colony. *Bull. Ent. Res.* 37: 619-27.
- ROBERTS, F. H. S. (1940).—The insect parasites of sheep. *Qd. Agric. J.* 53: 530-46.
- SKUSE, F. A. A. (1888).—Diptera of Australia. Part IV.—The Simuliidae and Bibionidae. *Proc. Linn. Soc. N.S.W.* 3: 1363-86.
- SKUSE, F. A. A. (1890).—Diptera of Australia. Nematocera—Supplement II. *Ibid.* 5: 595-640.
- SMART, J. (1945).—The classification of the Simuliidae (Diptera). *Trans. R. Ent. Soc. Lond.* 95: 463-528.
- TAYLOR, F. H. (1918).—Studies in phlebotomic Diptera, No. 1. New species of Simuliidae and Chironomidae. *Aust. Zool.* 1: 167-70.
- TAYLOR, F. H. (1927).—A note on *Simulium bancrofti* Taylor, with the description of a new species of *Simulium* (Dipt.). *Bull. Ent. Res.* 18: 70-2.
- TAYLOR, F. H. (1944).—Sandflies. *Aust. Mus. Mag.* 8: 210-3.
- TONNOIR, A. L. (1923).—Notes sur la biologie des larves de *Simulium* (Diptera). *Ann. Biol. Lacust.* 11: 163-72.
- TONNOIR, A. L. (1925).—Australasian Simuliidae. *Bull. Ent. Res.* 15: 213-55.

EXPLANATION OF PLATES 1-2

Plate 1

Fig. 1.—Breeding ground of *S. ornatipes* and *S. nicholsoni*. Biloela district.

Fig. 2.—Logs on which *S. ornatipes* were ovipositing abundantly, Don R., Calliungal. A former breeding ground of *A. pestilens*.

Plate 2

Fig. 1.—Breeding ground of *A. bancrofti*, Barambah Creek, near Gayndah. Flow almost fast enough for *A. pestilens*.

Fig. 2.—A former breeding ground of *A. pestilens*, Dawson River. These tea-trees were covered with empty pupal shells and cocoons.

INACTIVATION OF GONADOTROPHINS

I. INACTIVATION OF SERUM GONADOTROPHIN BY INFLUENZA VIRUS AND RECEPTOR-DESTROYING ENZYME OF *VIBRIO CHOLERAE*

By W. K. WHITTEN*

[Manuscript received April 2, 1948]

Summary

Serum gonadotrophin was rapidly inactivated by preparations of receptor-destroying enzyme from *Vibrio cholerae* and also by allantoic fluid from chick embryos which had been infected with adapted LEE-B strain of influenza virus.

A mucinase preparation from *V. cholerae* free from receptor-destroying enzyme did not affect the hormone, nor did normal chick allantoic fluid to which was added triturated chorioallantois.

Receptor-destroying enzyme or virus preparations when administered at the same time as serum gonadotrophin but at a remote site, significantly reduced the response to the latter.

It is concluded that the receptor-destroying activity of influenza virus and of cholera enzyme is responsible for the inactivation of serum gonadotrophin.

I. INTRODUCTION

Hirst (1941) observed agglutination of red cells by strains of influenza virus. During this reaction the virus was adsorbed on the red cells which, if present in sufficient numbers, thus removed the virus from the supernatant fluid. Later, Hirst (1942) observed that the virus was eluted during incubation of red cells which had been agglutinated with the virus. The red cells from which the virus had thus been removed were insusceptible to further agglutination by the same or fresh virus. However, the freed virus could be adsorbed on and eluted from successive batches of red cells without appreciable loss. Heated virus was also found to agglutinate red cells but was not freed on incubation.

From these experiments Hirst concluded that the virus acted as an enzyme, the substrate of which was the virus "receptor substance" on the surface of the red cell. This conclusion was supported by the findings of Burnet, McCrea, and Stone (1946) that a similar modification of red cells was produced by filtrates of *Clostridium welchii* and *Vibrio cholerae* cultures. These workers also observed that this action was common to the viruses of the mumps-influenza group in which it occurred with a gradient of activity.

Stone (1947) purified the cholera filtrate and found that the active substance exhibited all the characteristics of an enzyme for which the term receptor-destroying enzyme was adopted.

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Burnet, McCrea, and Anderson (1947) observed that glandular mucins and blood-group substances A and O inhibited the agglutination of red cells by heated virus and that this inhibition was prevented by incubating these substances beforehand with receptor-destroying enzyme. Burnet (1947) showed that influenza virus also removed the inhibiting activity of these substances thus confirming the similarity between influenza virus and receptor-destroying enzymes. However, the serological specificity of these mucins was not affected by these enzymes. A general account of the relationship between influenza virus, receptor-destroying enzyme, and mucopolysaccharides is given by Burnet (1948).

In his classification of mucoids and mucopolysaccharides Meyer (1945) has grouped gonadotrophic hormone and blood-group substances together as neutral mucoids containing acetylglucosamine and galactose. This similarity suggested an investigation of the effect of receptor-destroying enzyme on gonadotrophins.

The enzymic destruction of serum gonadotrophin has been examined by several workers. It was shown by Goss and Cole (1931) that trypsin and pepsin inactivated the hormone. These observations have been confirmed by Evans and Hauschildt (1942), who also showed that the hormone was readily attacked by papain, chymotrypsin and carboxypeptidase, and concluded that the hormone is susceptible to proteolytic enzymes in general.

McShan and Meyer (1938) observed rapid inactivation of serum gonadotrophin by human saliva. This observation was confirmed by Evans and Hauschildt (*loc. cit.*) who also found that taka-diastase destroyed the hormone. These workers concluded that α -amylase was responsible for the inactivation and that the hormone contained " α -hexoside" linkages in the polysaccharide groups. Evans and Hauschildt (*loc. cit.*) also observed slow inactivation of the hormone by emulsin whereas previously Cartland and Nelson (1937) observed none with that preparation nor with invertin.

The observations of Evans and Hauschildt (*loc. cit.*) using saliva and taka-diastase were re-examined and confirmed by the author (Whitten 1947*b*). However, two other highly active α -amylase preparations from malted barley and parotid salivary glands of rats were without effect on the hormone and it was concluded that the action of saliva and taka-diastase was not due to amylase.

In the same preliminary report inactivation of serum gonadotrophin by receptor-destroying enzyme was recorded. However, Burnet* states that human saliva contains only a trace of this enzyme which he considers to be the product of micro-organisms. This indicates that another mucolytic enzyme in saliva may be responsible for inactivation of the hormone. It is suggested that Schiff's blood-group enzyme may be active in this regard. We have obtained evidence, however, that neither lysozyme nor hyaluronidase has any effect on serum gonadotrophin. So far as the author is aware there are no other relevant observations of the effects of other enzymes on serum gonadotrophin.

The present communication deals with the inactivation of serum gonadotrophin by receptor-destroying enzyme and influenza virus *in vitro* and some

* Personal communication.

observations on the effect of the substances *in vivo* on the response to serum gonadotrophin. The control procedures adopted are also described.

II. MATERIAL AND TECHNIQUE

Gonadotrophic Preparation.—Commercial serum gonadotrophin (Organon) from sealed glass ampoules was dissolved in borate buffer pH7 just prior to the commencement of each experiment. No standard preparation was available for standardization of hormone used.

Enzyme Preparations from V. cholerae.—These preparations were kindly supplied by Professor F. M. Burnet. Dried receptor-destroying enzyme prepared as reported by Stone (loc. cit.) was dissolved in distilled water to make a 1 in 500 solution. This preparation contained a trace of the mucinase described by Burnet and Stone (1947). A sample of dried mucinase prepared from the same source but free from the receptor-destroying enzyme was used as a control in a 1 in 500 solution.

Influenza Virus.—Allantoic fluid of high titre from chick embryos which had been infected with adapted LEE-B influenza virus was also supplied by Professor F. M. Burnet. This fluid was used as the active virus preparation. Similarly treated allantoic fluid from normal chick embryos of the same age, to which was added triturated chorioallantoic membrane was used for control experiments. This was considered to resemble very closely the infected allantoic fluid, since influenza virus produces some necrosis of the chorioallantois.

Hormone Assays.—The animals used were female Wistar rats from a partially inbred strain, bred in this laboratory. At the commencement of each experiment they weighed 40-50 g. and were 23-25 days old. Litter mates were distributed equally between the groups and this distribution was randomized in respect to weight-ranking within each litter. A single injection of the hormone preparation was made and the animals were killed after 120 hours. The vaginae were examined for patency and the ovaries were removed and fixed in Bouin's solution overnight. They were then transferred to 70 per cent. alcohol, dissected, and weighed on a torsion balance, accurate to 1 mg. In all but one experiment, groups of 10 or more rats were used for each dose and the results were expressed as the mean ovarian weights. Each mean is accompanied by the standard error of that mean.

III. OBSERVATIONS

(a) *In vitro Inactivation of Gonadotrophin*

Spontaneous loss of activity of serum gonadotrophin in solution has been observed by Rimington and Rowlands (1944) and others. The procedure adopted was designed to eliminate any error arising from this source and also from augmentation or protection which may occur when substances are added to or injected together with gonadotrophins.

Experiments were conducted in which a volume of hormone solution, together with one-fifth of the volume of receptor-destroying enzyme or virus preparations, was incubated at 37°C. for 4 to 6 hours. In each case a duplicate control was prepared and treated in the same manner, with the exception that heat-inactivated enzyme or virus was used. Equal doses of these test and control mixtures were injected into respective groups of rats. The results (Table 1, experiments 1, 2, 3, and 4) show that both receptor-destroying enzyme and influenza virus completely inactivated the hormone.

Similar experiments were carried out using the mucinase from *V. cholerae* or normal allantoic fluid with triturated chorioallantois. From the results (Table 1, experiments 5 and 6) it is evident that no significant change in the hormone content occurred.

TABLE 1
EFFECT OF ENZYME AND VIRUS PREPARATIONS *IN VITRO* ON SERUM GONADOTROPHIN

Experiment No.	Enzyme Preparation	No. of Rats	Mean Ovarian Wt. (mg. \pm s.e.)	No. of Vaginae Open
1	Receptor-destroying enzyme	10	15 \pm 1	0
	Receptor-destroying enzyme (heat-inactivated)	10	56 \pm 4	10
2	Receptor-destroying enzyme	10	16 \pm 1	0
	Receptor-destroying enzyme (heat-inactivated)	10	52 \pm 5	10
	Receptor-destroying enzyme (injected at a remote site)	10	43 \pm 4	10
3	Influenza virus	12	18 \pm 1	0
	Influenza virus (heat-inactivated)	12	52 \pm 3	12
4	Influenza virus	11	17 \pm 1	0
	Influenza virus (heat-inactivated)	11	36 \pm 3	11
5	Mucinase	10	59 \pm 5	10
	Mucinase (heat-inactivated)	10	56 \pm 4	10
6	Allantoic fluid	10	83 \pm 4	10
	Allantoic fluid (heat-treated)	10	89 \pm 5	10
	Untreated animals	10	16 \pm 1	0

In experiment 2, an additional procedure was adopted to determine whether the inactivation occurred *in vitro* or was the result of inhibition of the ovarian response to the hormone by the active substance. The experiment was carried out as described above but hormone and enzyme were incubated separately and then injected at different sites into a third group of rats in doses equivalent to those received by the other two groups.

The results are given in Table 1, experiment 2, and show that total inactivation occurred *in vitro* whereas no significant difference was produced when the enzyme was administered at a remote site at that dose rate.

(b) *Action of Enzymes on Gonadotrophin in vivo*

Three groups of animals were injected with equal doses of serum gonadotrophin. At the same time and also eight hours later, one group received a subcutaneous injection of 1 ml. of the preparation of receptor-destroying enzyme. The second group was given an equivalent amount of material which had been held at 67°C. for 30 minutes, whereas the third group received no further injections. The response was observed by the usual assay procedure. The mean ovarian weights are recorded in Table 2. These results show that the ovarian response was completely inhibited by the active enzyme. It is difficult to understand the reduction in ovarian response caused by injection of the heated enzyme but since no check was made on the efficiency of heat inactivation no conclusions can be drawn.

TABLE 2

EFFECT OF RECEPTOR-DESTROYING ENZYME AND VIRUS *IN VIVO* ON RESPONSE TO SERUM GONADOTROPHIN

Experiment No.	Treatment	No. of Animals	Mean Ovarian Wt. (mg. \pm s.e.)	No. of Vaginae Open
1	Serum gonadotrophin and receptor-destroying enzyme injected separately	11	16 \pm 1	2
	Serum gonadotrophin and receptor-destroying enzyme (heated) and injected separately	11	75 \pm 5	11
	Serum gonadotrophin	11	125 \pm 11	11
2	Serum gonadotrophin and virus injected separately	8	78 \pm 12	8
	Serum gonadotrophin	8	145 \pm 13	8
	Untreated	10	16 \pm 1	0

A similar experiment was conducted using influenza virus, except that an injection of heated virus was omitted. The results, also given in Table 2, show a reduction in response which, however, was not as marked as with the enzyme.

IV. DISCUSSION

Burnet and his co-workers have established an almost complete parallelism between the action of influenza virus and the receptor-destroying enzyme of *V. cholerae* on red-cell receptors and mucins, including blood-group substances

A and O. In view of the fact that preparations of virus and enzyme destroy serum gonadotrophin, it appears certain that the same enzymic mechanism is involved. However, these findings require formal proof by the use of highly purified materials.

Many micro-organisms produce receptor-destroying enzymes. It may therefore be that bacterial contamination is responsible for the erratic behaviour of serum gonadotrophin in solution.

The inhibition of the action of serum gonadotrophin by simultaneous administration of virus or receptor-destroying enzyme resembles that observed by Whitten (1947*a*) with a fraction of human saliva when it was concluded that the inactivation of the gonadotrophin took place within the body. It is suggested that the virus and receptor-destroying enzyme may act in the same manner.

Inhibition of the response to serum gonadotrophin by other gonadotrophic extracts has frequently been observed. This field has been reviewed by Deanesley (1939) who also made several observations on this phenomenon. She concluded that this inhibition probably depended on the different rates of action of the hormones on the ovary. However, she observed that inert pituitary extracts also inhibited the action of serum gonadotrophin and suggested that a direct action between the two may be possible. The similarity between these reactions and those observed with influenza virus and enzymes warrants further investigation.

Elucidation of the mode of action of receptor-destroying enzymes should help to determine some of the essential groups of gonadotrophins.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- BURNET, F. M. (1947).—*Aust. J. Sci.* **10**: 21.
BURNET, F. M. (1948).—*Lancet* **1948**(I): 7.
BURNET, F. M., MCCREA, J. F., and ANDERSON, S. G. (1947).—*Nature* **160**: 404.
BURNET, F. M., MCCREA, J. F., and STONE, J. D. (1946).—*Brit. J. Exp. Path.* **27**: 228.
BURNET, F. M., and STONE, J. D. (1947).—*Aust. J. Exp. Biol. Med. Sci.* **25**: 219.
CARTLAND, G. F., and NELSON, J. W. (1937).—*J. Biol. Chem.* **119**: 59.
DEANESLEY, R. (1939).—*J. Endocrinol.* **1**: 307.

- EVANS, J. S., and HAUSCHILDT, J. D. (1942).—*J. Biol. Chem.* **145**: 335.
- GOSS, H., and COLE, H. H. (1931).—*Endocrinology* **15**: 214.
- HIRST, G. K. (1941).—*Science* **94**: 22.
- HIRST, G. K. (1942).—*J. Exp. Med.* **76**: 195.
- McSHAN, W. H., and MEYER, R. K. (1938).—*J. Biol. Chem.* **126**: 361.
- MEYER, K. (1945).—*Advances in Protein Chemistry* **2**: 247.
- RIMINGTON, C., and ROWLANDS, I. W. (1944).—*Biochem. J.* **38**: 54.
- STONE, J. D. (1947).—*Aust. J. Exp. Biol. Med. Sci.* **25**: 137.
- WHITTEN, W. K. (1947*a*).—*Aust. J. Sci.* **10**: 49.
- WHITTEN, W. K. (1947*b*).—*Ibid.* **10**: 83.

CORRIGENDA

VOLUME 1, NUMBER 1

Page 9, line 4 from bottom: *For* 15° *read* 5° .

Wardrop and Dadswell, Plate 2: Figure 1 and Figure 2 have been transposed.

Page 58, last line of *Summary*: *For* direct *read* indirect.

THE WOOD ANATOMY OF THE PROTEACEAE

By M. MARGARET CHATTAWAY†

[Manuscript received May 28, 1948]

Summary

This paper contains a general description of the wood anatomy of 26 genera of the Proteaceae. The genera described are those which produce timber trees or large woody shrubs. A map is given to show the geographical distribution of the woody genera of the Proteaceae. The relationship of the wood anatomy to the botanical classification is discussed, with special reference to those genera in which there are marked anatomical differences within the genus.

The wood anatomy is discussed in relation to that of other woods with large rays and the means of distinguishing between them are detailed.

I. INTRODUCTION

The family Proteaceae includes about 54 genera and over 1,000 species. The members consist of shrubs and trees, very rarely herbs, usually with spirally arranged, more or less leathery leaves, subdivided or entire, without stipules. The flowers are often clustered in large spikes or heads; the fruit is a dehiscent capsule or follicle, or an indehiscent stone fruit or nut (Ewart 1930). Most members of the family are markedly xerophytic.

The family shows two main centres of development: Australia where over 600 species occur, many genera of which are endemic, and South Africa which has about 300 species. The family extends from eastern Asia, China, and Japan down through Malaya to New Guinea. Members are also found in New Caledonia, Tahiti, New Zealand, and South America.

The family is subdivided, according to Engler and Prantl (1889) as follows:

A. PERSOONIOIDEAE

1. Persoonieae *Bellendena*, *Symphyonema*, *Agrastachya*, *Garnieria*, *Dilobeia*, *Beauprea*, *Cenarrhenes*, *Persoonia*,* *Brabeium*.*
2. Franklandieae *Franklandia*.
3. Proteeae *Isopogon*, *Petrophila*, *Sorocephalus*, *Nivenia*, *Serruria*, *Mimetes*, *Spatalla*, *Adenanthos*, *Faurea*,* *Protea*,* *Leucospermum*, *Leucadendron*,* *Aulax*, *Simsia*.
4. Conospermeae *Synaphea*, *Conospermum*.

B. GREVILLOIDEAE

1. Grevilleae *Darlingia*,* *Buckinghamia*, *Grevillea*,* *Carnarvonina*,* *Hakea*,* *Orites*,* *Helicia*,* *Xylomelum*,* *Lambertia*, *Roupala*,* *Panopsis*,* *Macadamia*,* *Hicksbeachia*,* *Kermadecia*, *Guevina*,* *Euplassa*.
2. Embothrieae *Embothrium*,* *Telopea*,* *Lomatia*,* *Knightia*,* *Cardwellia*,* *Stenocarpus*,*
3. Banksieae *Banksia*,* *Dryandra*,*

The genus *Musgravea* is now placed between *Darlingia* and *Buckinghamia*, and *Finschia* is placed near *Grevillea*.

† Division of Forest Products, C.S.I.R.

* Genera with species producing trees or large shrubs.

The family contains few plants of economic value, and most of the species are small or medium sized shrubs. Many, however, are handsome flowering trees well adapted for ornamental plantations, while nearly all are rich honey yielders and hence of value to apiarists. A few representatives form large trees, some of which produce valuable timber. Timbers of the silky oaks *Cardwellia sublimis*, *Orites excelsa*, and *Grevillea robusta* are used extensively in furniture and paneling. Satin oak (*Embothrium wickhami*) is used for the same purposes and also *Knightia excelsa* (rewa rewa of New Zealand). *Banksia serrata* has been used for boat knees, bullock yokes etc., and *B. verticillata* is used for carriage finishing etc. of railways in Western Australia as well as for furniture. *Hakea leucoptera* and *H. vittata* have been used for ornamental turnery and smoking pipes.

Of the extra-Australasian genera, *Faurea*, *Protea*, and *Leucadendron* occur from tropical to South Africa, and in Madagascar, but only *Faurea* is of commercial importance as a timber (Sim 1921; Scott 1927, 1935). *F. macnaughtoni* is a furniture wood and *F. saligna* can be used as a constructional timber. *Embothrium*, *Guevina*, *Lomatia*, *Panopsis*, and *Roupala* occur in the New World but do not grow to a great size; only *Roupala* is of any importance though the other genera are used locally for furniture etc. (Record and Hess 1943).

The accompanying map (Fig. 1) shows the geographical distribution of the woody members of the family according to Gardner (1941-2).



Fig. 1.—Map showing geographical distribution of the woody members of the Proteaceae.

Key to Genera (in alphabetical order):

- | | | |
|-----------------------|-------------------------|------------------------|
| 1. <i>Banksia</i> | 10. <i>Guevina</i> | 19. <i>Orites</i> |
| 2. <i>Cardwellia</i> | 11. <i>Hakea</i> | 20. <i>Panopsis</i> |
| 3. <i>Carnarvonia</i> | 12. <i>Helicia</i> | 21. <i>Persoonia</i> |
| 4. <i>Darlingia</i> | 13. <i>Hicksbeachia</i> | 22. <i>Protea</i> |
| 5. <i>Dryandra</i> | 14. <i>Knightia</i> | 23. <i>Roupala</i> |
| 6. <i>Embothrium</i> | 15. <i>Leucadendron</i> | 24. <i>Stenocarpus</i> |
| 7. <i>Faurea</i> | 16. <i>Lomatia</i> | 25. <i>Telopea</i> |
| 8. <i>Finschia</i> | 17. <i>Macadamia</i> | 26. <i>Xylomelum</i> |
| 9. <i>Grevillea</i> | 18. <i>Musgravea</i> | |

II. DESCRIPTION OF WOOD ANATOMY OF THE PROTEACEAE

(a) General Properties

The truewood of timbers of the Proteaceae varies in colour from a very light creamy brown (e.g. many *Persoonia* and *Helicia* spp.) through those tinted with pink (*Stenocarpus sinuatus*), pink-brown, and red-brown to the very deep red-brown tinted with purple of *Xylomelum occidentale*; sapwood is light coloured and contrasts sharply with the heartwood in those timbers which have deeply coloured heartwood. Pink and red tinted timbers are commonest in this family. Some timbers (e.g. *Cardwellia sublimis* and *Embothrium wickhami*) are very lustrous and this feature in company with others makes them highly suitable for use in furniture and interior finish.

In weight there is a range from the light timbers of *Cardwellia* and *Embothrium* with air-dry densities of 36 and 34 lb./cu.ft. respectively to *Hakea leucoptera*, a hard heavy timber with an air-dry density of 60 lb./cu.ft.

All timbers of this family, except some species of *Persoonia*, are characterized by a more or less well-marked ray figure due to the presence of broad rays which may, as in *Xylomelum*, reach a height of over 2 cm. and a width of over 1 mm. and form about 50 per cent. of the substance of the wood (Plate 1, Fig. 1). Vertical and intercellular canals (probably of traumatic origin) have been observed in *Grevillea robusta*, *G. striata*, *G. stenobotrya*, *Hakea macraena*, and *Musgravea stenostachya*; they have been reported in *Banksia* and *Cardwellia* (Record 1936; Record and Hess 1943).

(b) The Tissues

(i) *Vessels*.—The pore arrangement in the family varies from predominantly solitary (*Faurea macnaughtoni*, Plate 4, Fig. 6; Plate 2, Fig. 2) and solitary and short tangential and radial multiples (*Roupala dielsii*, Plate 1, Fig. 3) to a marked tangential arrangement (*Knightia excelsa*, Plate 1, Fig. 5). This tangential pattern is often closely connected with the arrangement of the parenchyma, and the two together, as seen on the cross section, may form festoons which extend tangentially from ray to ray, and are looped with the concavity towards the periphery (*Telopea oreades*, Plate 1, Fig. 4). The tangential bands of vessels may be only one or two deep radially as in *Knightia* (Plate 1, Fig. 5), or massed into wide bands which form the dominant pattern of the wood, as in many species of *Banksia* and *Hakea* (Plate 1, Fig. 6; Plate 5, Fig. 6). Other pore patterns occur within the family, though less commonly: an irregular scattered distribution of solitary or grouped pores as in *Cardwellia* (Plate 2, Fig. 1) and an irregular formation of tangential bands as in *Leucadendron argenteum* (Plate 3, Fig. 4). The vessels may vary much in size from the few large vessels of *Cardwellia* and *Carnavonia* to the small, closely ranked and quite uncountable vessels of *Banksia* and *Hakea*. Differences in pore size are common throughout the growth rings and are sufficiently marked in some species of *Hakea*, and occasionally in *Persoonia toru*, for the woods to be classed as semi-ring-porous. Microscopically the vessel elements have some features that serve to distinguish the genera. Although simple perforation plates predominate throughout the family, reticulate and

foraminate multiple perforation plates are common in the smaller vessels of *Lomatia* and *Telopea* (Plate 2, Fig. 3; Plate 6, Fig. 3). Spirals occur in *Banksia*, *Hakea*, *Dryandra*, *Grevillea*, and *Guevina*, but are not invariably present and are more conspicuous in some species than in others (Plate 2, Fig. 4). The inter-vessel pits vary in size from genus to genus; pits to parenchyma and ray cells are similar to the inter-vessel pits; in *Musgravea* and *Faurea* they are commonly, and in *Helicia*, *Panopsis*, and *Stenocarpus* occasionally, unilaterally compound in the parenchyma walls. Brown resinous deposits are common in the vessels of some species of *Banksia*, *Grevillea*, *Xylomelum* etc., and white deposits are sometimes conspicuous in the pores of *Cardwellia sublimis*. Tyloses have not been observed.

(ii) *Parenchyma*.—The parenchyma is commonly plentiful, apotracheal or paratracheal or both; three types of parenchyma arrangement can be distinguished within the family, though there is some overlapping between the types. *Reticulate parenchyma*, fine narrow lines, 1-3 cells wide, running from ray to ray and forming a typical reticulate pattern on the cross section, occur in *Musgravea*, *Hicksbeachia*, *Panopsis*, and some species of *Stenocarpus* and *Helicia* (Plate 3, Figs. 1, 2, and 6). In all these woods the parenchyma lines appear to be apotracheal and the narrow lines are approximately parallel and continuous. The vessels and vessel groups are rather few and lines of parenchyma are often completely independent of the vessel groups for a considerable distance across the wood. Vasicentric parenchyma is commonly also present around the vessel groups. *Banded parenchyma* (probably paratracheal), associated with the vessels and vessel groups distinguishes the majority of the other genera, and is characteristically on the abaxial side of the pores and pore groups. The parenchyma may be in narrow lines 1-3 cells wide (*Helicia ferruginea*, *Finschia* sp., Plate 3, Figs. 3 and 5) or bands of 4 and more cells wide (*Xylomelum pyriforme*, *Grevillea robusta*, Plate 1, Fig. 2; Plate 5, Fig. 2). Where the parenchyma lines are narrow and numerous, as in *Hicksbeachia* sp., *Darlingia*, and *Finschia* (Plate 3, Fig. 5), the fundamental distinction between this type and the reticulate is a little obscure, though the distinction is clear to the eye, and it is possible that the linking of every parenchyma line with one or more vessel groups and the absence of independent parenchyma lines is due to the greater number of vessel groups and not to a fundamental difference in the parenchyma type. It serves, however, as a useful diagnostic feature for distinguishing the woods (cf. Plate 3, Figs. 5 and 6). The lines and bands of parenchyma tend to curve from one large ray to another, and this, with the arrangement of the pore groups on the axial side of the parenchyma, gives the "festoon" or "pendant" pattern so characteristic of many Proteaceae. *Paratracheal parenchyma*, vasicentric and associated with apotracheal lines, or aliform and confluent to give irregular bands. This is characteristic of *Cardwellia*, some species of *Stenocarpus*, *Carnarvon* (where it is sometimes associated with diffuse parenchyma), *Xylomelum*, and *Embothrium wickhami* (where it is predominantly aliform) (Plate 5, Fig. 3). It is also common in the species which have lines of reticulate parenchyma and, in *Helicia*, its presence aids the distinction between the two groups into which the species can be divided.

(iii) *Rays*.—Large rays are characteristic of most genera, and it is only in the section *Persoonioideae* that really small rays occur. In this section, however, the rays are very variable in size and all the genera examined show species with some very large rays. In *Persoonia* the seven species examined showed a series from *P. quinquenervis*, with uniseriate rays, to *P. linearis* and *P. elliptica* in which the rays are of two distinct sizes and resemble closely those of some of the *Grevilloideae*. In *Faurea* (three species) there was again a gradual difference between the species, *F. macnaughtoni* approaching very closely the structure of some of the *Grevilloideae*. In the *Grevilloideae* the rays are commonly of two distinct types, large multiseriate rays and small uniseriate ones. It appears unlikely that the uniseriate rays all increase in size and become multiseriate, but the increase in ray tissue which is made necessary by the increasing perimeter of the stem seems to be brought about, as in the *Sterculiaceae* (Chattaway 1937) either by the formation of new uniseriate rays through the change from fusiform cambial initials into ray initials, or by the subdivision of multiseriate rays through the reverse change taking place. In some genera this subdivision produces only large rays, but in others (notably those of the *Banksia* type) the rays seldom attain such enormous proportions and the subdivision results in a graded series of rays, all distinct from the uniseriate rays, but often only 5 or 6 cells wide and not very high. The uniseriate rays are often formed only of erect cells, and in many woods are only 1 or 2 cells high (*Banksia grandis*, Plate 2, Fig. 5). The rays of *Banksia* and *Dryandra* are characterized by the presence of vascular tissue. On tangential sections these appear to have the structure of small stems, and on radial sections vessels and tracheids can be seen running radially along the rays. Some of these have the characteristic pitting and thickening of protoxylem elements (Plate 7, Figs. 1-4), others resemble small vessels and have simple perforation plates. Further details of this tissue will be published in a subsequent paper. Stone cells occur in the rays of species of *Persoonia* (Plate 6, Fig. 1), *Faurea*, *Stenocarpus* (Plate 6, Fig. 2), *Roupala*, and *Hakea* and have been observed in one species of *Grevillea* and one of *Panopsis*. Crystals are present in the stone cells of *Stenocarpus salignus* and in the ray cells of some species of *Hakea*.

(iv) *Fibres*.—In most genera the ground mass of the wood is formed of libriform fibres with simple or indistinctly bordered pits. In some species of *Stenocarpus*, and in *Xylomelum*, *Persoonia*, *Protea*, and *Faurea*, however, the ground mass of the wood is formed of fibre tracheids with conspicuously bordered pits. The walls are thin to moderately thick in the majority of the genera; where they are thick, the thickness of the wall is much greater than the diameter of the lumen. Septa have not been observed.

(v) *Tracheids*.—These occur in some genera, and appear to be of three different kinds: (i) tracheids of independent origin and probably the only true tracheids within the family; (ii) tracheids which are variants of the ground mass of fibre tracheids; (iii) tracheids which are variants of the small angular vessel segments.

In *Macadamia* the ground mass of the wood is formed of libriform fibres, but bands of tracheids occur at irregular intervals, possibly delimiting the growth

zones. This feature serves to distinguish *Macadamia* from all other members of the family examined (Plate 6, Figs. 5 and 6).

In some species of *Stenocarpus* and in *Xylomelum*, tracheids occur around and among the vessels and seem to be a development from the ground mass of fibre tracheids. In the vicinity of the vessels the fibre tracheids tend to have thinner walls and wider lumina and more numerous pits with large borders. In macerated material differences in length can be observed from the long fibre tracheid which forms the ground mass of the wood to the typical blunt-ended tracheid which is always associated with vessels or groups of vessels and which is identical in length with the vessel members.

In many of the genera with the *Banksia* type of structure there is great variation in vessel size, the smaller ones being little larger in diameter than the surrounding fibres and parenchyma cells. Among these small elements all gradations will be found from small vessel members with minute perforation plates, to imperforate elements which are indistinguishable from true tracheids. Tracheids of this type have also been found in *Persoonia*.

III. RELATION OF WOOD ANATOMY TO BOTANICAL CLASSIFICATION

The family Proteaceae has been divided into two groups, the Persoonioideae and the Grevilloideae. The wood anatomy suggests that though some members of the Persoonioideae are quite distinct from the members of the Grevilloideae variations in structure can be found within this group, leading to the structure characteristic of most of the Grevilloideae.

Persoonia quinquenervis is unlike any other member of the Proteaceae in having small solitary vessels, often independent of any parenchyma or capped abaxially by a few cells, and low predominantly uniseriate rays. Within this genus there is, however, a series leading to *P. elliptica* which has irregular bands and clusters of vessels, backed by irregular bands of parenchyma, and rays of two distinct sizes, with the large rays subdividing in the manner typical of other members of the family. In *Faurea* the structure approaches that of the Grevilloideae.

Within the Grevilloideae two main types of structure stand out, but these do not separate the genera according to the botanical classification given above; they actually split several of the genera, but they appear to be constant and serve usefully in separating the woods according to the following key:

KEY

Type 1. Parenchyma lines apotracheal, reticulate, *Musgravea*, *Helicia* (in part), *Panopsis*, *Stenocarpus* (in part).

Type 2. Parenchyma lines or bands paratracheal.

(2a) Vessels usually surrounded by parenchyma and often in the middle of wide bands of parenchyma, *Carnarvonia*, *Xylomelum*, and occasionally *Cardwellia* and *Grevillea*.

(2b) Vessels on the axial side of parenchyma lines or bands, solitary or in clusters, or tangential multiples, *Cardwellia*, *Darlingia*, *Embothrium* (in part), *Finschia*, *Grevillea*, *Helicia*, *Hicksbeachia*, *Macadamia*, *Roupala*, *Stenocarpus* (in part).

- (2c) Vessels on the axial side of the parenchyma lines or bands; in tangential arrangement, usually from ray to ray, often forming bands several vessels deep, *Banksia*, *Dryandra*, *Embothrium* (in part), *Guevina*, *Hakea*, *Knightia*, *Lomatia*, *Telopea*.

In Type 1 the parenchyma is arranged predominantly in close apotracheal lines, usually 2 or 3 cells wide, and the vessels do not occur solely on the axial side of the parenchyma as in the rest of the family, but are usually surrounded by a thin layer of vasicentric parenchyma. The fibres have rather thin walls in all the genera examined. It is occasionally a little difficult to distinguish between this type and woods such as *Hicksbeachia* in which the lines of paratracheal parenchyma are very numerous and narrow, but the size and position of the vessels, the vasicentric parenchyma and the thinner walled fibres should give the necessary clues. The genus *Helicia* serves to show both types of structure, the division of the genus in this respect being also noted by Janssonius (1934). A similar division of *Stenocarpus* has been noted by the author.

Type 2 is based on woods with paratracheal parenchyma which may be mainly aliform (as in *Embothrium*), but is more commonly confluent, forming narrow lines or broad bands which curve from ray to ray, concave towards the periphery, the vessels and vessel groups seeming to depend from the parenchyma as pendants from a chain. The pendant vessel arrangement is often obscure in Type (2a) where the vessels are sometimes completely surrounded by vasicentric parenchyma and even by the wide bands of parenchyma or by irregularities in confluent bands. It is sometimes obscure also in Type (2c) where the pattern is given to the wood on cross sections by the massed bands of vessels which tend to obscure the narrower lines of parenchyma.

In all except three genera the wood is, in spite of variations from species to species, and sometimes even within a species, of the same fundamental type. But in *Helicia*, *Embothrium*, and *Stenocarpus* there are very distinct and apparently fundamental differences, which might, if found also in the flowers and leaves, warrant division of each of these genera.

Helicia

H. ferruginea and *H. glabriflora* examined by the author, *H. serrata*, *H. attenuata*, and *H. javanica* described by Janssonius (1934), and *H. formosana* figured by Kanehira (1940) have wood of the typical *Grevillea* type, with small vessels, solitary or in clusters, appearing on cross sections as pendants from the parenchyma lines, touching the parenchyma only on the abaxial side, moderately numerous to numerous; parenchyma in narrow concentric paratracheal lines; rays often over 5 but less than 10 mm. high; fibres with very thick walls and small lumina and few simple pits (Plate 3, Fig. 3).

H. diversifolia and *H. montana* examined by the author and *H. incisa* and *H. lanceolata* described by Janssonius (1934) are of the *Musgravea* type, with vessels which are almost always surrounded by a narrow rim of vasicentric parenchyma, and are fewer and larger than in the other species; the parenchyma lines are apotracheal but are less numerous and less regular than in *Musgravea*; the rays usually less than 5 mm. high; the fibres thin walled, with small indistinctly bordered pits (Plate 3, Fig. 6).

According to Janssonius (1934) a division of the genus on these anatomical points is in accordance with the classification of Koorders and Valetton (1900) in their "Flora Javanica."

Embothrium

E. wickhami from Australia has a quite distinct structure from the three American species examined and it seems possible that these should be distinct genera. The wood shows clusters of vessels which lack the characteristic tangential pattern; the parenchyma is aliform and confluent, when confluent tending to form broad bands which enclose the vessel groups (Plate 5, Figs. 3 and 4).

Of the three American species, *E. coccineum* and *E. grandiflorum* show structure of the *Banksia* type, with bands of pores extending tangentially from ray to ray, often several pores deep radially with the parenchyma on the abaxial side of the bands. In the one available specimen of *E. weberbaueri* the tangential arrangement is less marked and this species seems (as in some species of *Banksia* and *Hakea*) to link the others to the *Grevillea* type. The pores of all three species are smaller and more numerous than those of *E. wickhami* and the rays smaller, lower, and less conspicuous.

Stenocarpus

The distinction between the two groups in *Stenocarpus* is similar to that observed in *Helicia*, but *Stenocarpus laurinus*, *S. reticulatus*, and *S. salignus*, which have woods of the *Grevillea* type, show a tendency for the parenchyma to be aliform as well as in regular concentric bands. In *S. salignus* there is a considerable variation from aliform parenchyma to broad bands which may enclose the pores (Plate 2, Fig. 6).

In *S. sinuatus* and *S. umbellatus* the wood is of the *Musgravea* type with clustered, irregularly spaced pores surrounded by vasicentric parenchyma and also with numerous fine concentric lines of apotracheal parenchyma (Plate 3, Figs. 1 and 2).

IV. DESCRIPTION OF THE GENERA*

A. PERSOONIOIDEAE

(a) *Faurea*

A genus consisting of about 15 species confined to tropical South Africa and Madagascar (Sim 1921; Scott 1927, 1935). Four species were available for examination: *F. discolor* Welw., *F. macnaughtoni* Phillips, *F. saligna* Harv., and *F. speciosa* Welw. (see Plate 2, Fig. 2, and Plate 4, Figs. 3, 5, and 6).

(i) *General Properties of the Timber*.—Light brown to dark brown in colour, moderately heavy (*F. macnaughtoni* 52-60 lb./cu.ft. air-dry†; cutting moderately evenly, rather fine in texture. Match-size splinters burn to a full white or buff ash.

(ii) *Structure*.—*Growth rings* not marked. *Pores* mostly solitary, sometimes in short chains and clusters but with a tendency to tangential arrangement; white deposits sometimes present; pits to parenchyma and ray cells sometimes unilaterally compound. *Parenchyma* usually abundant, in close association with the pores, occasionally vasicentric (Scott 1927) but more often aliform and confluent,

* In alphabetical order.

† Density figures given in this paper are on the air-dry basis at 12 per cent. moisture content.

on the abaxial sides of the pores, forming 1-8 seriate bands, sometimes broken, but sometimes continuous from ray to ray, sometimes anastomosing and somewhat concave towards the periphery. *Rays* large except in *F. speciosa* where they are usually not more than 5 mm. high; stone cells of very irregular shape have been observed in the large rays of one sample of *F. speciosa* and in *F. discolor*. *Fibre* wall thickness slightly greater than the diameter of the lumen; pits with small but distinct borders. *Tracheids* (and small imperfectly perforated vessel members) often associated with the vessel groups.

(b) *Leucadendron*

Only one species was available for examination, *L. argenteum* R.Br. The genus consists of about 70 species, confined to South Africa. *L. argenteum* is the best known and is cultivated, not for its timber but for the ornamental flowers and silvery silky leaves from which it gets its common name of "silver tree" (Plate 3, Fig. 4).

(i) *General Properties of the Timber*.—Light brown in colour and moderately light in weight. Fine and uniform in texture. Match-size splinters burn to a full dark grey ash.

(ii) *Structure*.—*Growth rings* not clearly defined but sometimes marked by slight differences in pore size. *Pores* moderate in size and moderately numerous; in pore multiples and clusters, seldom solitary, commonly arranged in loose concentric somewhat irregularly spaced bands. *Parenchyma* inconspicuous with a lens, paratracheal, sometimes, but not regularly on the abaxial side of the pore groups, forming bands 1-3 cells wide. *Rays* variable in size but seldom more than 1-5 mm. high, of all sizes from 1-10 cells wide. *Fibre* wall thickness much less than the diameter of the lumen; pits bordered but the borders small and indistinct.

(c) *Persoonia*

A genus consisting of shrubs and small trees which, with the exception of a single New Zealand species, is limited to Australia, which has 60 species. Seven species have been examined, but only one sample was available of each, viz. *P. elliptica* R.Br., *P. lanceolata* Andr., *P. longifolia* R.Br., *P. linearis* Andr., *P. media* R.Br., *P. quinquenervis* Hook., and *P. toru* A.Cunn. (Plate 5, Fig. 5; Plate 6, Fig. 1).

(i) *General Properties of the Timber*.—Sometimes tinged with pink or red, bark varying from brown and fine textured in some specimens to thick and flaky and red. Moderately light to heavy in weight, with air-dry densities of from 33-58 lb./cu.ft. (67 lb./cu.ft. in *P. quinquenervis*). Texture fine and even; match-size splinters burn to a full or partial buff or black ash.

(ii) *Structure*.—*Growth rings* not clearly defined. *Pores* from 20 to more than 50 per sq.mm. not visible to the naked eye and not always clearly defined with a lens; solitary (*P. quinquenervis*) and in multiples of 2-6, with tangential arrangement in irregular concentric bands often with scattered pores and pore multiples between the bands; the typical proteaceous pattern with lines of pores curving from ray to ray and concave towards the periphery is absent from this

genus, and in some specimens the lines of pores may be convex, following the curve of the stem; spiral thickening was observed in all species except *P. longifolia*, but varied in frequency and distinctness in different species. *Parenchyma* associated with the pores, on the abaxial side of the pores and pore groups, not clearly visible with a lens and scarce in all species except *P. longifolia*. *Rays* variable in size in the different species; in *P. quinquenervis* uniseriate, very occasionally partially biseriate; in *P. lanceolata* and *P. media* usually 1-4 and in *P. toru* 1-6 wide, the larger rays uncommon in *P. media*; in *P. toru* the small rays are predominantly uniseriate and 1-3 cells high; *P. elliptica*, *P. linearis*, and *P. longifolia* have the more typical proteaceous rays of two distinct sizes. Stone cells were observed in the rays of *P. elliptica*, *P. lanceolata* (Plate 6, Fig. 1), *P. linearis*, and *P. longifolia*. *Fibre* wall thickness less than the diameter of the lumen; pits bordered except in *P. toru* and *P. quinquenervis*. *Tracheids* in all species examined, the larger vessels and vessel groups being surrounded by small elements which show all transitional stages between vessel elements and tracheids.

(d) *Protea*

The genus consists of about 75 species of small trees and ornamental shrubs, confined to South Africa and cultivated more for their flowers than for the timber. Three species were examined: *P. elliottii* C. H. Wright, *P. grandiflora* Thunb., and *P. lepidocarpon* R.Br.

(i) *General Properties of the Timber*.—The wood is light brown in colour with a pinkish tinge and moderately light in weight. Match-size splinters burn to a full or partial ash.

(ii) *Structure*.—*Growth rings* feebly marked by slight difference in pore size. *Pores* frequently solitary, occasionally in short tangential multiples, moderately numerous. *Parenchyma* moderately abundant, rather irregular in distribution, paratracheal, aliform and confluent, in 1-4 seriate bands. *Rays* of two distinct sizes, the larger rather low, usually less than 5 mm. high, with many uniseriate rays in *P. grandiflora* but varying from uniseriate to many cells wide in *P. lepidocarpon*. *Fibre* wall thickness less than the diameter of the lumen; pits numerous, bordered.

B. GREVILLOIDEAE

(a) *Banksia*

A large genus of about 50 species widely distributed through Australia, to which they are endemic. They vary in habit from shrubs to large trees; several grow to about 50-60 ft. and produce timber of commercial value. The woods of different species are very similar and no attempt has been made here to separate them. The following species were examined: *B. aemula* R.Br., *B. attenuata* R.Br., *B. collina* R.Br., *B. dentata* Linn., *B. ericifolia* Linn., *B. grandis* Willd., *B. ilicifolia* R.Br., *B. integrifolia* Linn., *B. latifolia* R.Br., *B. littoralis* R.Br., *B. marginata* Cav., *B. menziesii* R.Br., *B. serrata* Linn., *B. verticillata* R.Br. (Plate 1, Fig. 6; Plate 2, Figs. 4 and 5; Plate 7, Figs. 1-4).

(i) *General Properties of the Timber*.—Pink to a deep red-brown, sometimes with a purplish tinge, with interlocked and sometimes wavy grain; moderately light (29-50 lb./cu.ft.), moderately fine but of uneven texture. Match-size splinters burn to a full or partial ash. Vertical gum canals have been reported by Record (1936).

(ii) *Structure*.—*Growth rings* not defined. *Pores* not visible to the naked eye (except in *B. latifolia*) but distinct with a lens (except in some specimens of *B. integrifolia*, *B. marginata*, and *B. menziesii*); in crowded tangential bands, concentric, up to about 7 pores deep radially, often looped from ray to ray, concave towards the periphery; spiral thickenings sometimes conspicuous, but often faint and very sporadic in occurrence; deposits occasionally present in some species. *Parenchyma* in narrow lines or bands, 1-4 cells wide on the abaxial side of the pores. *Rays* often inconspicuous on the cross section owing to their colour being the same as that of the fibres, of two distinct types; the larger usually less than 5 mm. high, except in *B. aemula*, *B. ilicifolia*, and *B. grandis* (in the last-named often more than 10 mm. high), the smaller seldom more than 4 or 5 cells high and mostly only 1 or 2, composed entirely of upright cells (Plate 2, Fig. 5). *Vascular tissue* (see p. 283) observed in the rays of all species, but variable in frequency. *Fibre* wall thickness variable in different species, greater than the diameter of the lumen in *B. aemula*, *B. attenuata*, *B. grandis*, *B. ilicifolia*, and *B. menziesii*, and occasionally in *B. collina*, *B. dentata*, *B. latifolia*, *B. marginata*, and *B. serrata*.

(b) *Cardwellia*

A monotypic Australian genus, *C. sublimis* F.Muell., occurring in north Queensland, and attaining a height of 100-150 ft. with a diameter at breast height of about 4 ft. (Plate 2, Fig. 1).

(i) *General Properties of the Timber*.—Pink or light brown to reddish-brown, often with a golden sheen, moderately light (31-39 lb./cu.ft.); grain often interlocked; rather coarse and irregular in texture. Match-size splinters burn to a charcoal, without leaving any ash. Vertical gum ducts reported by Record (1936). It is often very difficult to distinguish between *Cardwellia sublimis* and *Grevillea robusta*, the best distinguishing features are the burning splinter test, in which *G. robusta* gives a full ash; the pore size, which is somewhat larger in *C. sublimis*; the presence of white deposits in *C. sublimis*; and the presence of spiral thickenings in *G. robusta*.

(ii) *Structure*.—*Growth rings* not defined. *Pores* usually large, clearly visible to the naked eye, few to moderately numerous, 3-6/sq.mm. in clusters and short tangential multiples, usually enclosed in the parenchyma bands, white deposits very common. *Parenchyma* abundant, vasicentric, aliform and confluent, forming broad bands up to 12 cells wide, commonly enclosing the pores. *Rays* of two distinct types, the larger usually more than 5 but less than 10 mm. high; the smaller uniseriate or often partially biseriate, up to 12 cells high, composed of erect, square and procumbent cells. *Fibre* wall thickness less than the diameter of the lumen, but rather variable.

(c) *Carnarvonia*

A monotypic Australian genus, the one species, *C. araliaefolia* F.Muell., occurs in the tropical mixed jungles of north Queensland.

(i) *General Properties of the Timber*.—Dark reddish-brown with a purplish tinge, moderately heavy (35-45 lb./cu.ft.), grain often interlocked, texture rather coarse and uneven. Match-size splinters burn to a full brown ash with smoke and a brown exudation streaming from the pores (Swain 1928).

(ii) *Structure*.—*Growth rings* not defined. *Pores* sometimes solitary but usually in clusters of tangential multiples; sometimes individually distinct to the naked eye, but more often only distinct with a lens, about 6-10 per sq.mm.; commonly filled with reddish-brown deposits. *Parenchyma* occasionally aliform, usually confluent, in broad bands 2-6 cells wide, sometimes surrounding the pores and pore groups but more often touching them on the abaxial side and partially surrounding them; apotracheal parenchyma abundant, diffuse, as scattered cells and short tangential lines. *Rays* of two distinct types, the larger usually more than 5 but less than 10 mm. high and up to 0.5 mm. wide; the smaller few, uniseriate, mainly composed of square or procumbent cells. *Fibre* wall thickness greater than the diameter of the lumen.

(d) *Darlingia*

Specimens from one species, *D. spectatissima* F.Muell. from Queensland, a tree attaining about 100 ft. in height and 18 in. diameter, were available for examination.

(i) *General Properties of the Timber*.—Truewood pink-brown, moderately heavy (44-47 lb./cu.ft.) texture rather coarse. Match-size splinters burn to a partial cream or grey ash.

(ii) *Structure*.—*Growth rings* not defined. *Pores* barely visible to the naked eye but individually distinct with a lens; about 6-10/sq.mm., predominantly solitary but occasionally in pairs or short tangential multiples, deposits not observed. *Parenchyma* in fine curved concentric bands on the abaxial side of the pores; 1-8 (commonly 2-4) cells wide; in one specimen occasional short tangential lines occurred without obvious connection with any pores. *Rays* of two distinct sizes, the large rays commonly over 5, but less than 10 mm. high, and about 0.25 mm. wide, rather uniform in size; the small ones uniseriate, of erect cells only. *Fibre* wall thickness rather variable but usually less than the diameter of the lumen; pits with small but distinct borders.

(e) *Dryandra*

A genus of 50 species confined to the south of Western Australia. Most of the species are small shrubs; only two, which attain tree size, viz. *D. floribunda* R.Br. and *D. nobilis* Lindl., were available for examination. The timber has no known uses. The wood is structurally indistinguishable from that of some of the smaller species of *Banksia*. Vascular tissue, similar to that in the rays of *Banksia*, was observed.

(f) *Embothrium* (in part see p. 286)

A genus which occurs in South America, with one species, *E. wickhami* Hill and F. Muell., in eastern Australia (Plate 5, Fig. 3). The wood of the Australian species is very different from that of the American, and it is possible that the genus is ill-defined. *E. wickhami* is a tree of about 70-90 ft. high with a diameter of 13-28 inches; it occurs in Queensland and New South Wales.

(i) *General Properties of the Timber*.—Pink-brown with a golden sheen, moderately light (about 31 lb./cu.ft. air-dry, Swain 1928), with slightly interlocked grain, coarse and uneven in texture. Match-size splinters burn to a dark buff ash (Swain 1928).

(ii) *Structure*.—*Growth rings* not defined. *Pores* just distinct to the naked eye, about 4/sq.mm., solitary or in short tangential multiples and clusters; red deposits filling many pores; white deposits sporadic, present in some samples. *Parenchyma* not very abundant, vasicentric, often forming eccentric or incomplete sheaths around the vessels, aliform with short tangential wings, occasionally, though rarely forming curved bands from ray to ray and giving the looped appearance typical of the Proteaceae. *Rays* of two distinct types, the larger seldom more than 5 mm. high, the smaller uniseriate, composed almost entirely of upright cells. *Fibre* wall thickness less than the diameter of the lumen.

(g) *Embothrium* (in part see p. 286)

Three American species were available: *E. coccineum* Forst., *E. grandiflorum* Lam., and *E. weberbaueri* Perkins (Plate 5, Fig. 4). There are considerable differences in structure between the two parts of the genus, *E. coccineum* and *E. grandiflorum* having the *Banksia* type of structure, whereas *E. weberbaueri* is transitional between this and the *Grevillea* type. The American species of *Embothrium* are shrubs and small trees distributed throughout the Andean region of South America. They produce an attractive good quality timber, but the smallness of the tree prevents it being of any commercial value (Record and Hess 1943).

(i) *General Properties of the Timber*.—Light greyish-brown with a high lustre, moderately heavy and hard with straight grain.

(ii) *Structure*.—*Growth rings* ill-defined, sometimes showing, microscopically, differences in pore size. *Pores* in clusters and short tangential multiples (*E. weberbaueri*); extending from ray to ray and forming continuous bands, often several pores deep radially (*E. coccineum*, *E. grandiflorum*); numerous, not visible to the naked eye, but distinct with a lens. *Parenchyma* in lines and bands 2-4 cells wide on the abaxial side of the pore groups and bands. *Rays* of two distinct sizes; the larger seldom more than 5 mm. high in *E. coccineum* and *E. weberbaueri* but often over 10 mm. high in *E. grandiflorum*, rather variable in width; the smaller uniseriate, often low, and composed of square or upright cells. *Fibre* wall thickness less than the diameter of the lumen.

(h) *Finschia*

A small to moderate sized tree from New Guinea; two species, *F. densiflora* C.T.W. and *F. ferruginea* C.T.W. (Plate 3, Fig. 5) were examined. The wood is very similar to that of some species of *Grevillea* but has thinner fibre walls, and, in some specimens, uniseriate rays composed predominantly of upright cells.

(i) *Grevillea*

A large genus of about 170 species of trees and shrubs distributed throughout Australia. *G. robusta* gives one of the timbers known as silky oak. The following species were available for investigation: *Grevillea barklyana* F.Muell., *G. heliosperma* R.Br., *G. hilliiana* F.Muell., *G. pinnatifida* F.Muell., *G. polystachya* R.Br., *G. robusta* A.Cunn., *G. stenobotrya* F.Muell., *G. striata* R.Br., and *G. subargentea* C.T.W. (Plate 4, Fig. 4; Plate 5, Fig. 2).

(i) *General Properties of the Timber*.—The heartwood is pinkish and lustrous, moderately light to rather heavy (37-66 lb./cu.ft., mostly 38-45 lb./cu.ft. air-dry), grain commonly interlocked; texture coarse and uneven. Match-size splinters burn to a full white or buff ash. Red deposits frequent in some specimens.

(ii) *Structure*.—*Growth rings* not defined, but occasionally there is a slight difference in pore size in early and late wood in some specimens of *G. robusta* and *G. stenobotrya*. *Pores* usually 6-10/sq.mm.; some solitary but usually in irregular clusters or short radial or tangential multiples, on the axial side of the parenchyma bands; vessel members sometimes with spiral thickening. *Parenchyma* predominantly in rather broad concentric bands, often looping from ray to ray, concave towards the periphery, 2-8 (commonly 4-6) cells wide, on the abaxial side of the pores and pore groups; often aliform and occasionally vasicentric; a few scattered cells or short tangential lines of diffuse parenchyma are sometimes present, especially in *G. robusta*. *Rays* of two distinct kinds, the larger variable in height and width, seldom more than 5 mm. high but sometimes up to 1 mm. wide, the small ones predominantly uniseriate, 5-15 cells high, usually of square or procumbent cells and very rarely of upright ones. *Fibre* wall thickness almost always greater than the diameter of the lumen except in some samples of *Grevillea pinnatifida*, *G. robusta*, and *G. subargentea*.

(j) *Guevina*

A monotypic genus, represented by *G. avellana* Molina from Chile. A small to medium-sized tree, occasionally growing to 65 ft. with a diameter of 30 in. The timber is used locally (Record and Hess 1943).

(i) *General Properties of the Timber*.—Heartwood pale brown with a pinkish tinge; lustre high but broken by the bands of pores and parenchyma; rather light; texture medium to rather coarse.

(ii) *Structure*.—*Growth rings* not defined. *Pores* indistinct to the naked eye but clearly visible with a lens, numerous, in clusters and tangential bands, irregular and parallel, curving from ray to ray, slightly concave towards the periphery; deposits not observed; vessel members with spiral thickening. *Parenchyma* in narrow bands 1-3 cells wide on the abaxial side of the pore bands. *Rays* of two distinct types, the larger commonly more than 5 but less than 10 mm. high; the smaller uniseriate, usually only 1-3 cells high, of upright cells only. *Fibre* wall thickness less than the diameter of the lumen.

(k) *Hakea*

A large Australian genus of about 100 species, mostly shrubs or small trees, seldom attaining a great size. The following species were available for examination: *H. cycloptera* R.Br., *H. eriantha* R.Br., *H. flexilis* F.Muell., *H. laurina*

R.Br., *H. lorea* R.Br., *H. leucoptera* R.Br., *H. macraena* F.Muell., *H. multilineata* Meissn., *H. pedunculata* F.Muell., *H. preisii* Meissn., *H. recurva* Meissn., *H. saligna* Knight, *H. subulata* A.Cunn., *H. vittata* R.Br. (Plate 5, Fig. 6).

(i) *General Properties of the Timber*.—Woods usually rather dark in colour, reddish-brown; sometimes rather heavy (46-60 lb./cu.ft.) and hard to cut, very fine but uneven in texture. Match-size splinters burn to a full white or buff ash. Vertical gum canals were observed in *H. macraena*.

(ii) *Structure*.—*Growth rings* often marked by a distinct difference in pore size between the beginning and end of the growth zone, sometimes amounting to definite semi-ring porosity. *Pores* usually visible, but sometimes indistinct with a lens; densely clustered in tangential bands from ray to ray, often several pores deep radially; commonly round in outline, with rather thick walls, except in *H. eriantha*, *H. laurina*, and *H. saligna*; in isolated clusters rather than in bands in *H. pedunculata*, *H. preisii*, and *H. subulata*; extremely numerous and very difficult to count; spirals sometimes very conspicuous, but not observed in all species, and often sporadic within a sample. *Parenchyma* sparse around the pores and usually in narrow bands on the abaxial side of the pore bands. *Rays* rather small and not very conspicuous in *H. laurina*, *H. lorea*, *H. subulata*, and *H. vittata*; of two distinct sizes, the larger usually less than 5 mm. high, varying in size and often with very thick walls to the individual ray cells, the smaller often rather few, sometimes of erect cells only; stone cells, often containing crystals observed in *H. laurina*, *H. leucoptera*, *H. recurva*, *H. subulata*, *H. vittata*; a few crystals were observed in ordinary ray cells in *H. pedunculata* and *H. preisii*. *Fibre wall thickness* much greater than the diameter of the lumen. *Tracheids*: all stages of transition can be found in the broad bands of vessels, between very small perfect vessels, small vessels with imperfect perforations, and imperforate vessel members that are difficult to distinguish from true tracheids (see p. 283).

(1) *Helicia* (in part see p. 285)

The genus is represented by about 50 species scattered throughout the Pacific Area from Australia to Japan. Two species of this section of the genus were available, *H. ferruginea* F.Muell. and *H. glabriflora* F.Muell. Both are Australian, but appear to be identical in structure with *H. serrata* Blume, *H. attenuata* Blume, and *H. javanica* Blume from Java, and to *H. formosana* Hemsl. from Formosa.

(i) *General Properties of the Timber*.—Light grey-brown with pinkish rays; moderately light (about 36 lb./cu.ft.); texture rather fine and uniform. Match-size splinters burn to a full grey ash.

(ii) *Structure*.—*Growth rings* not defined. *Pores* numerous (about 20/sq. mm.), not individually visible to the naked eye, but distinct with a lens; some solitary but predominantly in short tangential multiples on the axial side of the soft tissue; spirals absent from all specimens examined, though cited for this genus by Record (1936). *Parenchyma* in fine regular concentric lines from ray to ray, concave towards the periphery and on the abaxial side of the vessel groups; 1-4 (but usually 2) cells wide. *Rays* of two distinct sizes, the larger sometimes less than 5 mm. high in *H. ferruginea* but usually between 5 and 10 mm. high; the

smaller uniseriate, few and composed of erect cells only; a few small crystals were observed in the ray cells of *H. glabriflora*. Fibre wall thickness greater than the diameter of the lumen.

(m) *Helicia* (in part see p. 285)

The species with the *Musgravea* type of structure are described here; the specimens available were *H. diversifolia* C.T.W. and *H. montana* Sym. (Plate 3, Fig. 6). The trees are indistinguishable botanically from other species of the genus and are of similar distribution; species with this type of structure occur in Java as well as on the mainland of Australia.

(i) *General Properties of the Timber*.—The differences between these species and the rest of the genus lie chiefly in the distribution of the vessels and parenchyma and the thinner fibre walls.

(ii) *Structure*.—*Growth rings* not well defined. *Pores* few and scattered, solitary, clustered and in short radial and tangential multiples. *Parenchyma* paratracheal, vasicentric, surrounding the pores and pore groups; apotracheal, in concentric lines usually 1 or 2 cells wide. *Rays* of two distinct kinds, the large ones usually less than 5 mm. high, small ones uniseriate, few, commonly of erect cells only. *Fibre* wall thickness considerably less than the diameter of the lumen.

(n) *Hicksbeachia*

This is a monotypic genus from eastern Australia. *H. pinnatifolia* F.Muell., a small tree with edible nuts, provided material for examination.

(i) *General Properties of the Timber*.—Very light or straw-coloured, with conspicuous ray figure; moderately light (about 37 lb./cu.ft.), texture very fine but uneven owing to the large rays. Match-size splinters burn to a full grey ash.

This wood is structurally of interest as it is intermediate between woods such as *Helicia* and *Macadamia* with the *Grevillea* type of structure, and the *Musgravea* type (see p. 285). The fibres are very thick walled, the vessels small and on the axial side of the parenchyma lines as in the *Grevillea* type, but there are many parenchyma lines which seem entirely unconnected with any vessel or vessel group and suggest the development of lines of a definitely apotracheal type. Some of these lines are short and broken, giving diffuse parenchyma. The vasicentric parenchyma that accompanies the pore groups of the *Musgravea* type is entirely absent.

(ii) *Structure*.—*Growth rings* not defined. *Pores* indistinct even with a lens, solitary and in short multiples and clusters, on the axial side of the lines of parenchyma. *Parenchyma* abundant, as fine narrow lines 1 or 2 cells wide, irregularly concentric and often broken or anastomosing; some paratracheal on the abaxial sides of the vessels and vessel groups and some apparently apotracheal, without any connection with the vessels. *Rays* of two distinct sizes, the larger seldom more than 5 mm. high and up to 2 mm. wide, the smaller uniseriate, few, consisting entirely of upright cells. *Fibre* wall thickness greater than the diameter of the lumen.

(o) *Knightia*

The genus consists of 3 species, occurring in New Zealand and New Caledonia (Gardner 1941-2). Only one, *K. excelsa* R.Br. from New Zealand, was available for study. It forms a tree up to 100 ft. high, with a diameter of 3 ft. (Plate 1, Fig. 5; Plate 4, Fig. 1).

(i) *General Properties of the Timber*.—Light brown with darker and conspicuous ray fleck, moderately heavy (46 lb./cu.ft. air-dry), grain straight or slightly wavy. The wood is used for furniture veneers, as well as cabinet work, turnery etc. It is also in demand for brake shoes, and, being easily split, for shingles and fence rails.

(ii) *Structure*.—*Growth rings* not defined. *Pores* not visible to the naked eye but distinct with a lens, numerous; regularly arranged in tangential multiples, generally only 1 pore wide, forming concentric lines, curved and concave towards the periphery and extending from ray to ray. *Parenchyma* forming bands 2-3 cells wide on the abaxial side of the pores. *Rays* of two distinct types: the larger conspicuous on all faces though seldom more than 5 mm. high; the smaller up to about 5 cells high, formed predominantly of upright cells. *Fibre* wall thickness less than the diameter of the lumen.

The structure of *Knightia* is very similar to that of *Orites* (p. 296). Both can be separated from *Grevillea* by the straighter lines of pores and parenchyma and the more regular tangential arrangement of the pores from ray to ray.

(p) *Lomatia*

This genus with 13 species is widely distributed through eastern Australia, the Pacific islands, and South America. Four species were available for examination: *Lomatia dentata* R.Br. and *L. obliqua* R.Br. from Chile and *L. ilicifolia* R.Br. and *L. longifolia* R.Br. from Australia (Plate 2). The species are structurally indistinguishable. They grow to large shrub size or small trees, and the wood is only of local importance. In gross structure the wood is indistinguishable from *Guevina* (p. 292); microscopically, however, it can be distinguished by the absence of spiral thickenings from the vessels and the presence of irregularly reticulate multiperforate perforation plates (Plate 2, Fig. 3). If the origin of a specimen is known this too will serve to distinguish the genera.

(q) *Macadamia*

This genus of 5 species is confined to eastern Australia. Two species were available, *M. praealta* Bailey and *M. ternifolia* F.Muell. (Plate 6, Figs. 5 and 6).

(i) *General Properties of the Timber*.—Deep pinkish-brown; moderately heavy (44 lb./cu.ft. air-dry) with interlocked grain; said to have an unpleasant odour when green. Match-size splinters burn to a thin white ash.

(ii) *Structure*.—*Growth rings* not visible to the naked eye but defined microscopically by bands of tracheids (Plate 6, Fig. 6). *Pores* indistinct to the naked eye but visible with a lens, about 20-25/sq.mm., solitary and in short tangential multiples arranged rather regularly on the axial side of the lines of parenchyma. *Parenchyma* paratracheal, usually 2 cells wide in regularly spaced concentric

lines, on the abaxial side of the vessels and vessel groups. *Rays* of two distinct sizes, the larger usually more than 5 but less than 10 mm. high, the smaller, uniseriate, few, seldom more than 3 or 4 cells high, composed entirely of upright cells. *Fibre* wall thickness greater than the diameter of the lumen.

(r) *Musgravea*

The genus consists of one species endemic to Queensland, *M. stenostachya* F.Muell. (Plate 6, Fig. 4). It is one of the smaller of the jungle trees, about 60-80 ft. high. It has a limited range in north Queensland. The tough lobed leaves may be a foot or more in length (Swain 1928).

(i) *General Properties of the Timber*.—Heartwood brown and lustrous, moderately light in weight (30-37 lb./cu.ft. air-dry), texture fine and even, sometimes with vertical gum canals. Match-size splinters burn to a full grey ash.

(ii) *Structure*.—*Growth rings* not defined. *Pores* of the larger size just visible to the naked eye; 3 or 4/sq.mm., some solitary but mostly in clusters and short radial and tangential multiples. *Parenchyma* to some extent paratracheal and vasicentric, surrounding the pores and pore groups; but predominantly apotracheal, in narrow curved parallel bands, usually 2 or 3 cells wide, and about 4/mm. *Rays* of two distinct sizes, the large ones up to 5 mm. high and about 0.50 mm. wide, the uniseriates rather few and mostly of upright cells. *Fibre* wall thickness usually less than the diameter of the lumen; pits few, indistinctly bordered.

(s) *Orites*

This is a small genus of about 6 species confined to eastern Australia. One species was available for examination, *Orites excelsa* R.Br. (Plate 4, Fig. 2).

(i) *General Properties of the Timber*.—Light pink-brown with a golden sheen; moderately light (31 lb./cu.ft.), grain sometimes slightly interlocked; moderately fine but uneven. Match-size splinters burn to a full buff ash.

(ii) *Structure*.—The wood is structurally indistinguishable from that of *Knightia* (p. 295), but deposits were occasionally observed in the pores.

(t) *Panopsis*

The genus consists of a few species widely distributed through tropical South America. The trees are small to medium-sized; the best known, *P. rubescens* Pohl., attaining about 50 ft. The timber is used locally for cabinet work but is of no commercial importance (Record and Hess 1943). The only material available was *P. sessilifolia* (Rich) Sandwith and two samples of unnamed species.

(i) *General Properties of the Timber*.—Heartwood pinkish-brown with very conspicuous lighter coloured rays. Lustrous, varying in weight and hardness, with rather coarse and uneven texture given by the ray tissue.

(ii) *Structure*.—*Growth rings* not defined. *Pores* visible to the naked eye, about 2 or 3/sq.mm., some solitary, but mostly in clusters and short radial and tangential multiples. Pits to ray cells and parenchyma occasionally unilaterally compound. *Parenchyma* to some extent paratracheal, vasicentric surrounding the pores and pore groups, but predominantly apotracheal, in narrow concentric lines

usually 2 or 3 cells wide, somewhat convex from ray to ray, the concavity towards the bark, about 6-8/mm. Rays of 2 distinct sizes, the large ones often more than 10 mm. high and about 1-1.5 mm. wide, the uniseriate rays rather few and consisting almost entirely of upright cells. Fibre wall thickness less than the diameter of the lumen.

This timber is the only American member of the Proteaceae with this type of structure: *Andriapetalum* is now considered synonymous with *Panopsis*.

(u) *Roupala*

A genus of about 60 species, most abundantly represented in Central and South America, but also occurring in New Caledonia. Six species were examined: *R. angustifolia* Diels, *R. brasiliensis* Klotzsch, *R. complicata* Linden, *R. dielsii* Macbride, *R. loranthoides* Meissn., *R. montana* Aubl.; the wood structure of all is very similar (Plate 1, Fig. 3). *R. brasiliensis* is a rain-forest tree attaining a height of 100 ft. or more with a trunk 24-30 in. diameter and about 50 feet free of branches. The timber is highly durable and is employed locally in exposed structures, railway sleepers, and to a small extent for furniture and cabinet work (Record and Hess 1943).

(i) *General Properties of the Timber*.—Brown to reddish-brown, sometimes with a purplish tinge, sometimes lustrous, texture rather coarse. Match-size splinters burn to a full black ash.

(ii) *Structure*.—Similar to that of many species of *Grevillea* from which it can be distinguished microscopically by the finer texture, the presence of stone cells in the rays, and by the uniseriate rays which are low, usually 1 or 2 cells high and consisting entirely of upright cells.

(v) *Stenocarpus* (in part see p. 286)

A genus distributed through eastern Australia, New Guinea, and the Pacific Islands. Three species with the *Grevillea* type of structure were examined: *S. laurinus* Panch and Sebert, *S. reticulatus* C.T.W., *S. salignus* R.Br.; of these only *S. salignus* has any commercial value (Plate 2, Fig. 6; Plate 6, Fig. 2).

(i) *General Properties of the Timber*.—Red-brown, moderately heavy (about 50 lb./cu.ft.), grain wavy and interlocked. Match-size splinters burn to a full buff ash.

(ii) *Structure*.—Growth rings not defined. Pores variable in size in different species, in *S. laurinus* visible only with a lens, in the other two species with the naked eye, usually few (*S. reticulatus*) to moderately numerous, solitary or in short tangential multiples; white deposits sometimes observed in *S. reticulatus* and *S. salignus*. Parenchyma on the abaxial side of the pores, rarely surrounding them in *S. salignus*, predominantly aliform in *S. reticulatus* and in bands 3-7 cells wide in the other species. Rays of two distinct sizes, the larger commonly over 5 but usually under 10 mm. high, the smaller uniseriate, up to 15 cells high, of square and procumbent cells; stone cells commonly present in the large rays, sometimes containing crystals. Fibre wall thickness greater than the diameter

of the lumen; pits rather conspicuous, bordered. *Tracheids* present, probably transitional stages between true tracheids and fibres, around the vessels and sometimes at the limits of the growth rings; with larger lumina than the fibres and more numerous pits.

The wood of *S. laurinus* and *S. salignus* resembles *Grevillea* in its gross structure, but the two genera can be distinguished microscopically by the presence of stone cells in *Stenocarpus*, by the fibre pitting, and by the tracheids that accompany the vessels and vessel groups.

(w) *Stenocarpus* (in part see p. 286)

Two species with the *Musgravea* type of structure are described here: *S. sinuatus* Endl. and *S. umbellatus* Schlechter (Plate 3, Figs. 1 and 2). The structure of these two species of *Stenocarpus* is almost identical with that of the species of *Musgravea* described above (p. 296). Vertical gum canals have not been observed in *Stenocarpus*, but as these are not a regular feature of *Musgravea* their absence cannot be taken as indicative of *Stenocarpus*.

(x) *Telopea*

This is a genus of three species, confined to eastern Australia and Tasmania. Only one species, *T. oreades* F.Muell., was available for examination (Plate 6, Fig. 3). It is a small tree 30-40 ft. high and 1½-2 ft. in diameter. It is often cultivated for its ornamental flowers.

(i) *General Properties of the Timber*.—Light pink-brown, moderately light (39-46 lb./cu.ft.), grain interlocked, texture moderately fine. Match-size splinters burn to a buff ash.

(ii) *Structure*.—In gross structure the wood of *Telopea* is not distinguishable from that of *Guevina* (see p. 292). Microscopically, however, it can be distinguished by the absence of spiral thickenings from the vessels and the presence of irregularly reticulate multiperforate perforation plates. If the origin of a specimen is known this too will serve to distinguish the genera, as *Guevina* occurs only in South America.

(y) *Xylomelum*

This is a small genus of Australian shrubs and small trees. Three species were available for examination: *X. occidentale* R.Br., *X. pyriforme* Knight, and *X. salicinum* A.Cunn. (Plate 1, Fig. 1).

(i) *General Properties of the Timber*.—Dark red-brown, moderately light (about 40 lb./cu.ft. air-dry), very brittle, coarse and uneven in texture, with a marked figure due to the enormous rays. Match-size splinters burn with difficulty to a charcoal or to a charcoal stump and thin white ash.

(ii) *Structure*.—*Growth rings* not defined. *Pores* indistinct to the naked eye but distinct with a lens, about 5-8/sq.mm.; solitary and in clusters, sometimes tangentially aligned; usually with abundant reddish deposits and with sporadic white deposits in *X. pyriforme*. *Parenchyma* very abundant, aliform, and confluent in broad bands which may be up to 20 cells wide in *X. occidentale*, usually

enclosing the pores (*X. occidentale*), but sometimes (especially in the other two species) only on the abaxial side of the pores and partially surrounding them. *Rays* of two distinct types, the larger enormous (in *X. occidentale*, probably the largest in the Proteaceae and sometimes forming 50 per cent. of the bulk of the wood); the smaller up to about 16 cells high, composed almost entirely of square or procumbent cells. *Fibre* wall thickness of the walls greater than the diameter of the lumina; pits bordered. *Tracheids*, which are probably transitional stages between true tracheids and fibres, present around the vessels.

V. DISTINCTION BETWEEN PROTEACEAE AND OTHER FAMILIES WITH LARGE RAYS

The greatest distinguishing features of the Proteaceae are the large rays and the tangential pattern formed on the cross section by the parenchyma and vessels. Where this pattern is well marked it is sufficiently characteristic to distinguish the Proteaceae from all other woods with large rays. Where it is not so marked confusion may arise. This is most likely to occur between some of the *Grevillea* type, in which the predominance of aliform parenchyma, or broad bands containing the vessels, disturbs the characteristic "festoon" or "necklace" pattern. A macroscopic examination might not bring out the points of difference between *Cardwellia* and *Embothrium* and some of the Rhizophoraceae (for example *Carallia* and *Pellocalyx*). The scalariform perforation plates and pitting of the Rhizophoraceae will, however, serve to separate these two families without difficulty.

Hoheria (Malvaceae) is easily confused macroscopically with some of the Proteaceae of the *Banksia* type, although even with a lens it is possible to see that in *Hoheria* the parenchyma occurs on both sides of the curved pore bands and not, as in *Banksia* etc., on the abaxial side. Reference to a radial section does not give the clue to the identity as spiral thickenings occur on the vessel walls in both *Hoheria* and many of the Proteaceae of the *Banksia* type, but the storeyed parenchyma which can be seen on the tangential section of *Hoheria*, and the absence of uniseriate rays, should serve to distinguish them.

The *Musgravea* type of structure might be confused with some of the woods with reticulate parenchyma and large rays, but the ray width of the Proteaceae is usually sufficient to distinguish these woods. Where confusion might arise, as with a few of the Anonaceae, the characteristic proteaceous curving of the parenchyma from ray to ray should serve as a distinction, even though the characteristic "pendant" vessel pattern is absent.

In the few members of the family in which only small rays are present, there may be difficulty in distinguishing the woods, as the range of possible families is extended. Only by use of all available features and microscopic study can such woods be identified.

VI. KEY TO PROTEACEAE

A key for the identification of the Proteaceae based on the card sorting method has been prepared, and though designed primarily for use with a hand lens (x10) it is supplemented by a few features which can only be observed by

the use of a prepared slide and a microscope. It has not been found practicable to separate species, and each card represents a genus; where some species can be distinguished by the presence of any special feature a note has been added on the back of the card.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
		Semi-ring porous	Solitary	Clusters & Multiples	Tangential from ray to ray.	Broad bands	Very few	Moderately Numerous	Numerous	Large	Intermediate	Small	Very small	Deposits Common		Around the pores	Alliform occasionally	Confluent	On abaxial side of pores	Regular lines or bands.	Apothecial lines	Brood bands	Diffuse	
VESSELS															SOFT TISSUE									
GENUS																								
Species examined																								
RAYS					OTHER FEATURES										DISTRIBUTION									
26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
		Small & inconspicuous	Often > 10mm. high	Seldom > 5mm. high		Heavy	Intermediate	Light	Hard to cut.	Intermediate	Soft to cut.	Vertical gum canals	Burns to ash	Chars		Australia	New Caledonia & Pacific Islands	China & Japan	Malaya & E. Indies	New Zealand	S & C Africa	S & C America		

51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67
		Multiple perforation plates.	Spirals	Tracheids, bands.	Tracheids, ass'd vessels.	Conspicuous bordered pits.	Ray-vessel pits unilaterally compound	Stone cells in rays	Crystals (St. cells)	Crystals (ray cells)	Rays vary in width	Uniseriate - erect.	Vascular tissue in rays.	Fibre walls very thick.		
MICROSCOPIC FEATURES																

Fig. 2.—Front (upper) and back (lower) of card suggested for card sorting key to the Proteaceae.

As the principle lying behind the use of this type of key has already been explained (Clarke 1938) it will not be described in detail here, but a specimen card is shown in Figure 2. The details of the genera of the Proteaceae for use with such a card-sorting key can be extracted from the foregoing descriptions of the genera, or may be obtained from the author.

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VIII. REFERENCES

- CHATTAWAY, M. M. (1937).—*Philos. Trans. B* **228**: 313-66.
 CLARKE, S. H. (1938).—*New Phytol.* **37**: 369-74.
 ENGLER and PRANTL (1889).—"Die Natürlichen Pflanzenfamilien." (Wilhelm Engklmann: Leipzig.)
 EWART, A. J. (1930).—"Flora of Victoria." (Govt. Printer: Melbourne.)
 GARDENER, C. A. (1941-2).—*J. Roy. Soc. W. Aust.* **28**: xi-lxxxvii.
 JANSSONIUS, H. H. (1934).—"Mikrographie des Holzes, etc.," Vol. V. pp. 380-413. (E. J. Brill: Leiden.)
 KANEHIRA, R. (1940).—*Bull. For. Exp. Sta. No. 2.* (Govt. of Taiwan.)
 KOORDERS, S. H., and VALETON, T. (1900).—"Flora Arborea Javanica," Part V. (G. Kolf. & Co.: Batavia.)
 RECORD, S. J. (1936).—*Trop. Woods* No. 47: 12-27.
 RECORD, S. J., and HESS, R. W. (1943).—"Timbers of the New World." (Yale Univ. Press: New Haven.)
 SCOTT, M. H. (1927).—*S. Afr. J. Sci.* No. 24: 298-317.
 SCOTT, M. H. (1935).—*S. Afr. Dep. Agric. For. Bull. No. 145.* (For. Prod. Inst. Ser. I.) (Govt. Printer: Pretoria.)
 SIM, T. R. (1921).—*S. Afr. Dep. Mines and Industries, Mem. No. 3.* (Govt. Printer: Pretoria.)
 SWAIN, E. H. F. (1928).—"The Timbers and Forest Products of Queensland." (Govt. Printer: Brisbane.)

EXPLANATION OF PLATES 1-7

PLATE 1

- Fig. 1.—*Xylomelum pyriforme* Knight. Transverse surface of wood. x 10.
 Fig. 2.—*Xylomelum pyriforme* Knight. Transverse section. x 64.
 Fig. 3.—*Roupala dielsii* Macbride. Transverse section. x 64.
 Fig. 4.—*Telopea oreades* F.Muell. Transverse surface of wood. x 10.
 Fig. 5.—*Knightia excelsa* R.Br. Transverse section. x 64.
 Fig. 6.—*Banksia marginata* Cav. Transverse section. x 64.

PLATE 2

- Fig. 1.—*Cardwellia sublimis* F.Muell. Transverse surface of wood. x 10.
 Fig. 2.—*Faurea macnaughtoni* Phillips. Transverse section. x 75.
 Fig. 3.—*Lomatia ilicifolia* R.Br. Radial longitudinal section. Multiple perforation plate. x 400.
 Fig. 4.—*Banksia marginata* Cav. Radial longitudinal section. Spirally thickened vessel members. x 200.
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PLATE 3

- Fig. 1.—*Stenocarpus sinuatus* Endl. Transverse surface of wood. x 10.
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 Fig. 3.—*Helicia ferruginea* F.Muell. Transverse section. x 75.
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 Fig. 6.—*Helicia* sp. Transverse surface of wood. x 10.

PLATE 4

- Fig. 1.—*Knightia excelsa* R.Br. Transverse surface of wood. x 10.
 Fig. 2.—*Orites excelsa* R.Br. Transverse surface of wood. x 10.
 Fig. 3.—*Faurea saligna* Harv. Transverse surface of wood. x 10.
 Fig. 4.—*Grevillea robusta* A.Cunn. Transverse surface of wood. x 10.
 Fig. 5.—*Faurea speciosa* Welw. Transverse surface of wood. x 10.
 Fig. 6.—*Faurea macnaughtoni* Phillips. Transverse surface of wood. x 10.

PLATE 5

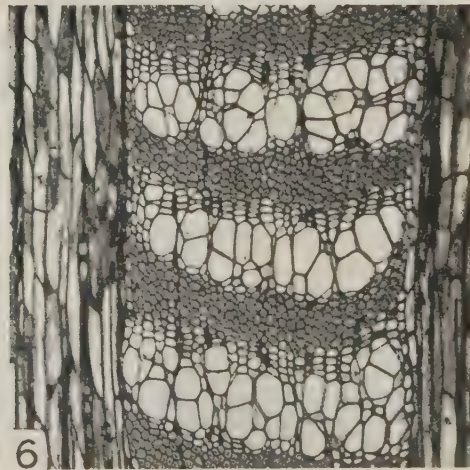
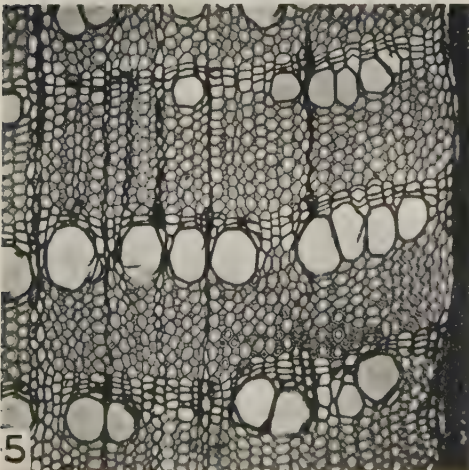
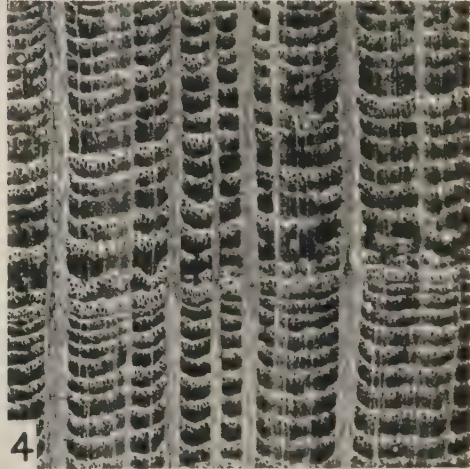
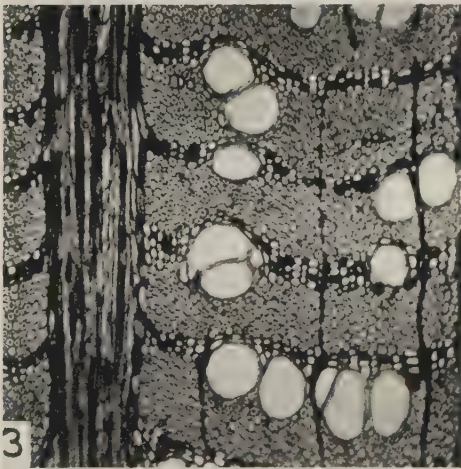
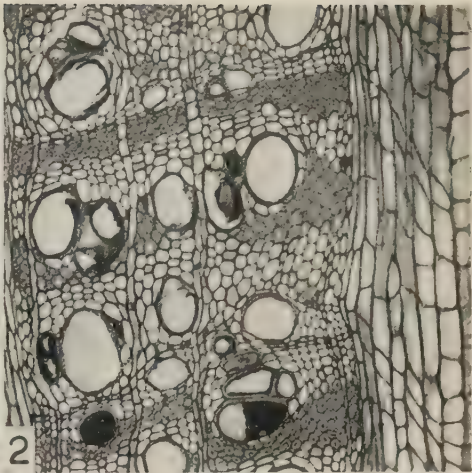
- Fig. 1.—*Protea lepidocarpon* R.Br. Transverse section. x 64.
 Fig. 2.—*Grevillea robusta* A.Cunn. Transverse section. x 64.
 Fig. 3.—*Embothrium wickhami* Hill and F.Muell. Transverse section. x 75.
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 Fig. 5.—*Persoonia longifolia* R.Br. Transverse section. x 75.
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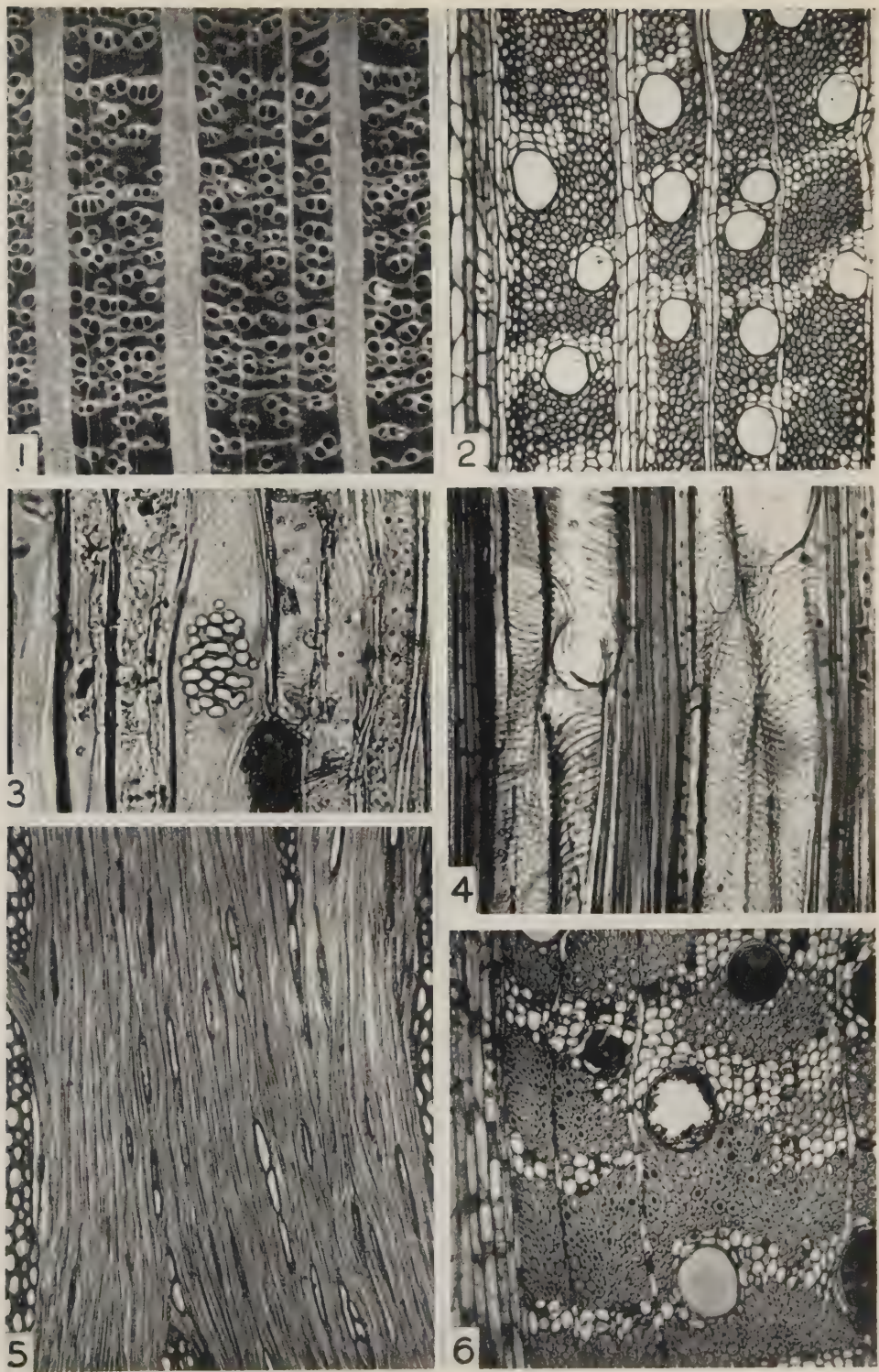
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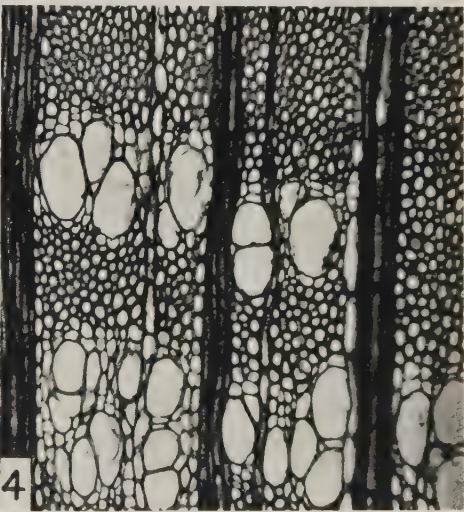
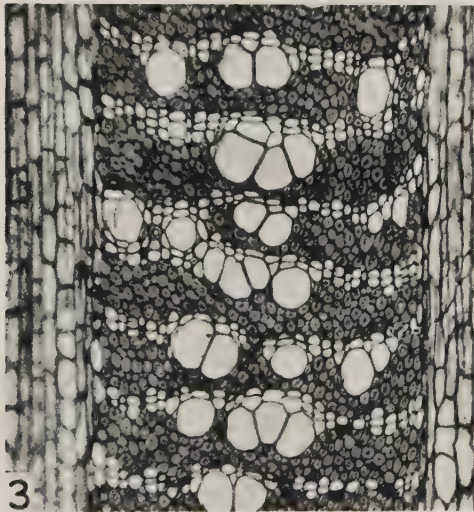
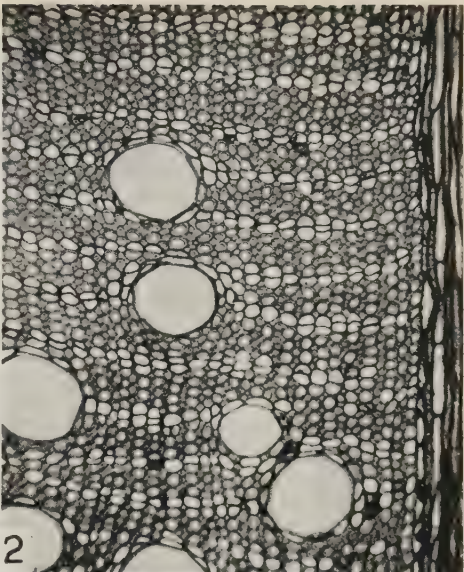
- Fig. 1.—*Persoonia lanceolata* Andr. Tangential longitudinal section. Stone cells in rays. x 75.
 Fig. 2.—*Stenocarpus salignus* R.Br. Tangential longitudinal section. Stone cells in rays. x 400.
 Fig. 3.—*Telopea oreades* F.Muell. Irregularly reticulate perforation plate. x 400.
 Fig. 4.—*Musgravea stenostachya* F.Muell. Transverse surface of wood. x 10.
 Fig. 5.—*Macadamia ternifolia* F.Muell. Transverse section showing band of tracheids. x 225.
 Fig. 6.—*Macadamia ternifolia* F.Muell. Transverse section. x 75.

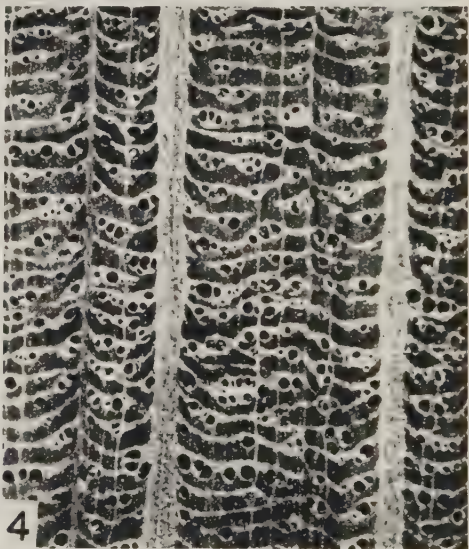
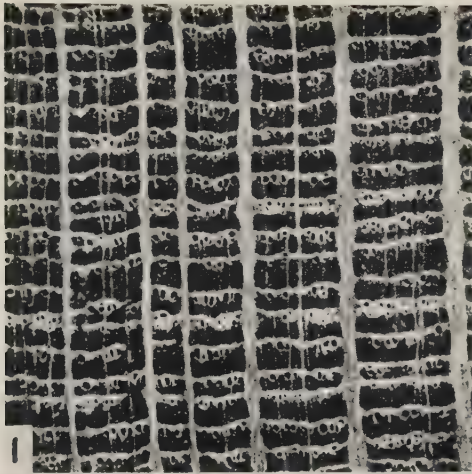
PLATE 7

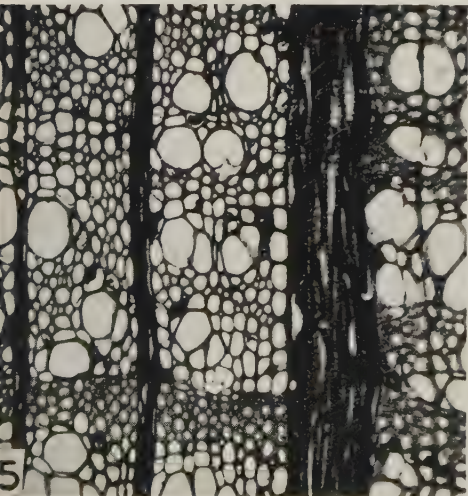
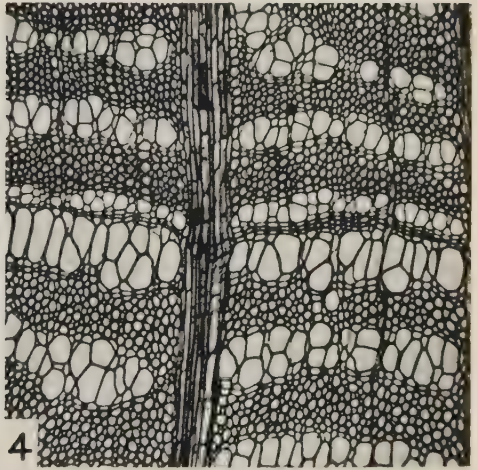
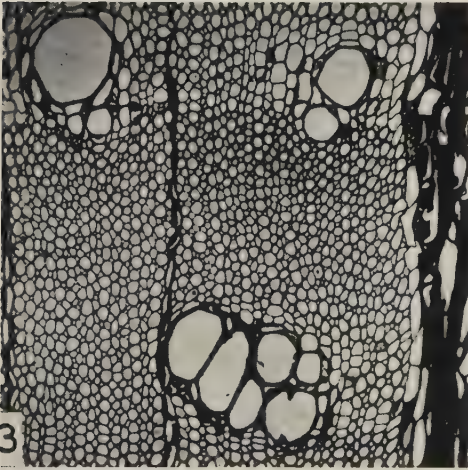
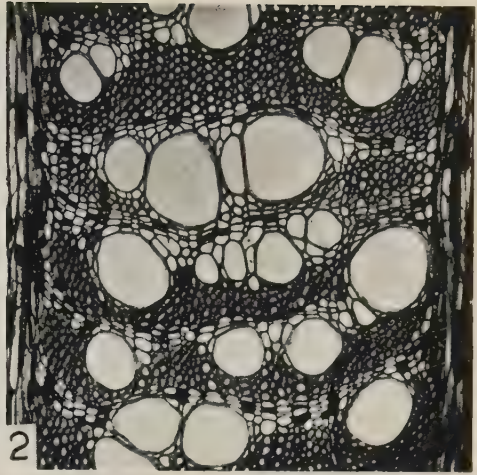
- Fig. 1.—*Banksia grandis* Willd. Tangential longitudinal section. Vascular tissue in large ray. x 65.
 Fig. 2.—*Banksia grandis* Willd. Radial longitudinal section of large ray, with vascular tissue. x 120.
 Fig. 3.—*Banksia ilicifolia* R.Br. Tangential longitudinal section. Vascular tissue in large ray. x 48.
 Fig. 4.—*Banksia grandis* Willd. Elements of vascular tissue from ray, isolated by maceration. x 140.

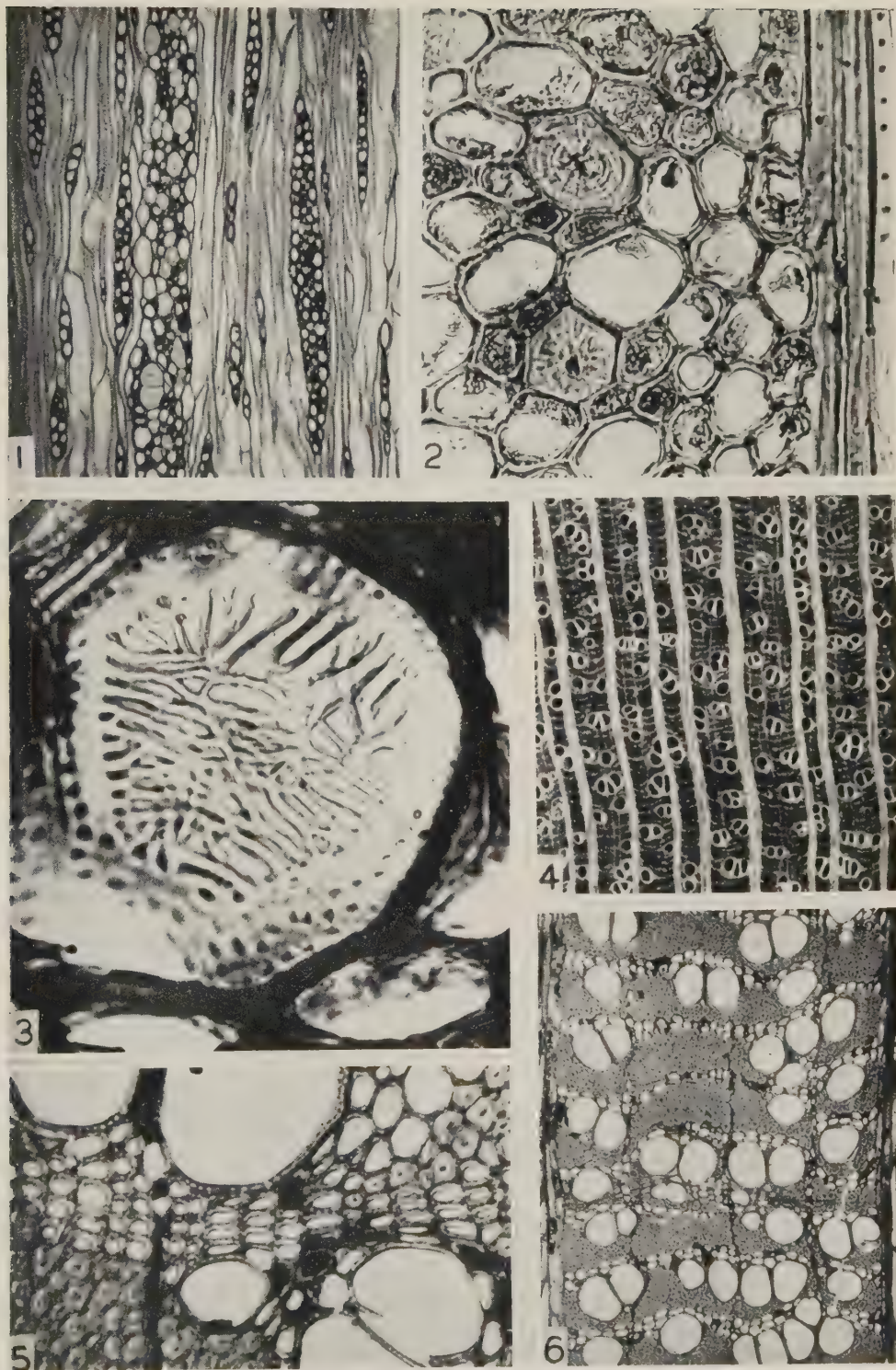


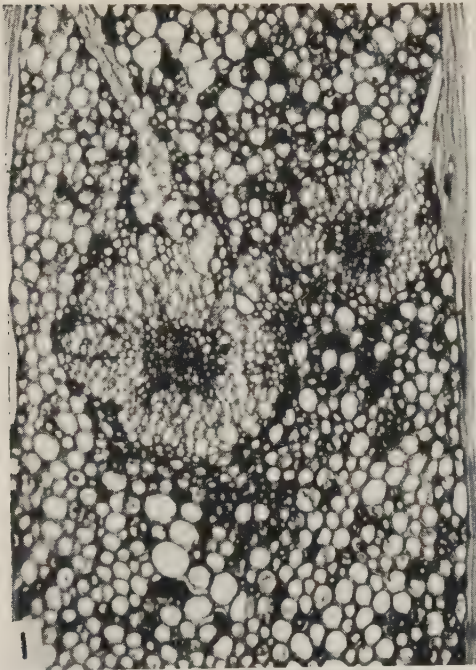












A NEW VIRUS DISEASE OF CARROTS: ITS TRANSMISSION, HOST RANGE, AND CONTROL

By L. L. STUBBS*

(Plates 1-4)

[Manuscript received May 26, 1948]

Summary

A new virus disease of carrots, which was recorded in Melbourne in 1943, is described. The disease occurs throughout Victoria, and has also been recorded in New South Wales, South Australia, Western Australia, and Tasmania.

The disease caused the virtual abandonment of early carrot production in the Melbourne vegetable area and, during the last war, was the principal limiting factor to carrot seed production.

In Victoria, carrot crops are exposed to infection during the period April to December, but a disease-free period occurs during the summer months.

Infected crops have a stunted, unthrifty appearance suggestive of mineral deficiency. Foliage symptoms consist of an irregular chlorotic mottle and marginal reddening of the lower leaves. Leaflets are distorted and reduced in size, and petioles and subpetioles are twisted longitudinally. Growth stunting is severe in susceptible varieties. Stem necroses sometimes develop on the youngest leaves of fully grown, recently infected plants, and on the older leaves when infection is of long duration.

The virus is transmitted by the aphid *Cavariella aegopodii*, which is a serious pest of carrots; but not by the aphids *Hyadaphis foeniculi*, *Anuraphis tulipae*, *Myzus persicae*, nor *Macrosiphum* spp. The Jassids, *Thamnotettix argentata*, *Erythoneura ix*, and *Empoasca* sp., also failed to transmit the virus.

The virus has been transmitted by core-grafting, but not by mechanical inoculation methods. There is no evidence of transmission through carrot seed.

Carrot is the only known natural host plant of the virus, but *Apium ammi*, *A. australe*, hemlock, dill, and coriander have been experimentally infected. Celery, parsley, parsnip, caraway, and fennel appear to be immune to the virus.

A wide range of cultivated varieties and wild strains of *Daucus carota* developed infection when grown under field conditions, but several local varieties were tolerant to the disease.

The vector, which is widely distributed in Victoria, occurs commonly on willow and fennel, and is a serious pest of carrot, celery, parsley, and parsnip.

Histological studies of the feeding habits of the vector showed that it was a phloem feeder.

The virus is of a persistent type and, in one experiment, the vector, after an infection feeding period of forty-eight hours remained infective during eighteen days of serial transfers. There appears to be no evidence of the occurrence of a latent period.

In two controlled experiments virus infected roots were subject to high mortality when transplanted for seed, whereas all healthy roots produced vigorous seed plants. Surviving infected seed plants lacked vigour and seed production was greatly reduced.

The invert sugar content of healthy roots was significantly higher than that of infected roots.

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Virus infected and healthy roots rotted at the same rate when wound inoculated with fungal organisms which were associated with root decay in Victorian seed crops.

It is believed that the biological breakdown of transplanted infected roots is frequently preceded by a form of necrotic collapse directly attributable to the virus.

Environmental control of the vector, and the disease, has been obtained by delaying the sowing of susceptible varieties until early summer.

In a spraying trial with spring sown Chantenay carrots, weekly applications of a proprietary DDT emulsion almost completely eliminated the vector, significantly reduced the amount of disease, and resulted in a sixfold increase in yield of marketable roots.

Disease control has also been demonstrated by comparing the infected yields of several virus tolerant varieties, and one susceptible variety, grown under experimental conditions. A Victorian virus tolerant selection out-yielded other varieties in this trial.

I. INTRODUCTION

During 1940 commercial vegetable growers in the Melbourne market-garden area requested an investigation of a disease of spring sown carrots, which had been occurring over a number of years. Summer sowings were not affected, but the failure, or partial failure, of spring sowings had made the production of this crop uneconomic, and as a result, the continuity of carrot supplies to the Melbourne market had been disrupted.

The principal objects of the investigation described in this paper have been the elucidation of the factors responsible for the disease, and the development of practical disease control measures.

A preliminary account of the investigation, which commenced in 1941, has been reported elsewhere (Stubbs and Grieve 1944).

II. HISTORY OF THE DISEASE

According to some experienced vegetable growers, spring carrot production was abandoned in many of the older suburban market-gardens about thirty years ago. The soil was said to have become "carrot sick" in these areas.

However, the disease was not reported until the spring of 1938 when crop failures occurred in the newer market-gardens on the outskirts of the city area. These sparsely settled districts probably owed their earlier freedom from the disease to their isolation, and it is a logical assumption that the disease followed the population trend from the inner to the outer suburbs as home gardens supply continuous reservoirs of infection.

During the last war, when carrot root and seed production was greatly increased, the disease appeared in isolated rural districts in which carrots were not formerly grown. It is thought that the disease was introduced to these districts by infected seed-roots brought from established vegetable areas.

In the early years of war-time seed production a high incidence of root-rot resulted in the failure of most carrot seed crops grown in Victoria. These failures were eventually attributed to the transplanting of infected roots, and were largely overcome by the selection of healthy root crops for seed production.

III. IDENTIFICATION OF THE DISEASE

In surveys of metropolitan market-gardens during the spring and summer of 1941-42 all spring sown carrot crops were found to be affected. These crops were uniformly stunted and had an unthrifty chlorotic appearance suggestive of a nutritional disorder.

The symptoms of the disease resembled those described by Warington (1940) for carrots grown in water culture medium deficient in boron. Her experiments were duplicated by the author, and the symptoms shown by boron deficient carrots were found to be very similar to those occurring in field crops.

At this stage of the investigation a lime-induced boron deficiency appeared to be a possible cause of the disease, and during 1941 and 1942 nutritional experiments were carried out with boron and other trace and major elements, but with negative results.

In the course of these experiments it was noticed that the appearance of disease symptoms was preceded or accompanied by infestations of various species of leaf-hoppers and aphids which, in view of the failure of the nutritional experiments, suggested the possibility of the disease being caused by an insect transmitted virus.

At that time virus diseases of carrots had only been recorded in the United States, where both the Aster Yellows virus (Zundel 1929; Severin 1930) and the Western Celery Mosaic virus (Severin and Freitag 1938) caused economically important diseases. Neither of these viruses had been recorded in Australia.

In preliminary virus transmission experiments, healthy carrot seedlings and various indicator plants (*Nicotiana tabacum*, *Datura stramonium*, etc.) were mechanically inoculated with sap expressed from diseased plants, but negative results were obtained.

In November 1942, insects occurring in diseased carrot crops were collected and identified; of these the Jassid *Thamnotettix argentata* Evans was a known vector of a virus disease (Hill 1941). During the summer of 1942-43 adult specimens of *T. argentata* were caged on the foliage of diseased carrots where they bred readily. The adult progeny of these Jassids were fed on the foliage of healthy carrots, but the plants remained healthy. Negative results were also obtained when similar transfers were made with the leaf-hoppers *Erythroneura ix* Myers and *Empoasca* sp.

Commencing on September 1, 1943, fortnightly sowings of carrots were made in an insect-proof field cage and concurrently in an area adjacent to this cage, at Burnley. The plants grown outside the cage became heavily infested with one species of aphid, and developed typical disease symptoms in November, whereas those grown within the cage remained vigorous and continued to produce normal foliage.

On November 20, 1943, groups of aphids from the affected plants were caged on single leaves of each of six semi-mature carrots within the field cage. The aphids were allowed to feed for seven days before being removed together with the leaves on which they had been feeding. Twenty-one days after the introduction of the aphids a mosaic mottle developed on the youngest fully

emerged leaves of these plants. Symptoms identical with those shown by the plants outside the cage soon appeared and, as the surrounding uninoculated plants remained healthy, the suspected virus origin of the disease was confirmed.

Specimens of the aphid used in this experiment were identified by E. H. Zeck, Department of Agriculture, N.S.W., and by the Imperial Institute of Entomology, London, as *Cavariella aegopodii* Scopoli.

IV. DISTRIBUTION

The disease has been recorded throughout Victoria and in New South Wales, South Australia, Western Australia, and Tasmania. The known distribution of the disease in Victoria is shown in Figure 1.



Fig. 1.—Known distribution of the carrot virus disease in Victoria.

V. SEASONAL OCCURRENCE

In all districts the disease cycle approximates very closely to the aphid cycle. The vector is favoured by the moist, cool conditions of spring and autumn, but it is unable to survive even the moderate summer temperatures of a normal season in southern Victoria. The aphid appears to be less sensitive to low temperatures, and light populations may persist on carrots, or other hosts, throughout the winter. However, a combination of low temperatures and heavy continuous rains is not conducive to its development. In the spring the vector is extremely active on many Umbelliferous species, and it is during this period, probably owing to the prevalence of winged forms, that virus spread is most rapid. In a plot of spring sown carrots it is almost impossible to record the rate of spread of the virus. The initial appearance of winged aphids is followed by the development of a few infection foci within a plot, and in a week or ten days symptoms appear almost simultaneously on the remainder of the plants. In the

autumn the aphids are more sluggish, winged forms are less prevalent, and the rate of virus spread is considerably reduced.

The duration of the infestation is dependent upon the climatic environment of the locality and upon current seasonal conditions. During two seasons (1943-44 and 1944-45) disease surveys showed that all carrot crops sown at Orbst and Bruthen prior to the first week in December became infected. In the drier Lindenow district November sowings were free from virus, but October sowings were heavily infected. In the metropolitan area carrots sown after the second week in December remained healthy. In north-western Victoria (Mildura, Swan Hill), where carrots are grown as a winter crop, an autumn and winter infestation is of more importance than a spring infestation: the latter is of relatively short duration because of the higher temperatures experienced in this area.

VI. SYMPTOMS

In spring or early summer an infected carrot crop has a uniformly unthrifty appearance which is more typical of a physiological disorder than of a virus disease.

The foliage of infected plants is dwarfed relative to that of healthy plants and the older leaves exhibit an irregular chlorotic mottle which may be accompanied by marginal reddening. These latter symptoms, which vary considerably from plant to plant, and according to the age of the plant at the time of infection, resemble the autumn tinting of deciduous plants (see Plate 1, Fig. 1).

The individual leaflets of infected plants are distorted, and reduced in size, and their surfaces are inclined in various planes due to longitudinal twisting of the petioles and subpetioles (see Plate 1, Fig. 2). The petioles of the older leaves are sometimes bent in an "S"-shaped manner (see Plate 1, Fig. 3).

With the advent of high temperature conditions, symptom expression gradually decreases in intensity, and foliar mottling may be completely masked. The writer, while searching for healthy crops for seed production purposes, experienced difficulty in detecting infected crops during the summer period. Symptom masking was most complete in dry-farming areas subject to hot dry conditions in midsummer.

When, however, a healthy crop grown under these conditions is viewed alongside an infected crop with masked symptoms, distinct differences in size and colour of foliage become apparent. Healthy carrot foliage of the Chantenay variety approximates closely in colour to a dark, dull yellow green and infected unmottled foliage to grass green or cress green (Ridgway 1912). The individual leaflets of infected plants are also considerably smaller than those of healthy plants. Unfortunately, these differences are of a relative nature and are, therefore, not reliable diagnostic aids.

Further evidence in support of the suggested relationship between air temperature and symptom masking is provided by the behaviour of infected plants grown under glass-house conditions. In winter, when maximum temperatures rarely exceed 70°F. and diurnal variations are of the order of 20-25°F., experimentally infected carrots develop intense foliage mottling, and are very dwarfed relative to healthy plants (see Plate 2, Fig. 1). In spring, summer, and

autumn, when maximum day temperatures frequently exceed 85°F., and the diurnal range is smaller, leaf mottling is difficult to detect, and dwarfing is less severe.

Mature summer-sown carrots, which usually become infected in autumn or early winter, develop a severe and characteristic reaction to the virus. The petioles of leaves emerging subsequent to infection are twisted, brittle, and reduced in length, and sometimes exhibit brown necrotic streaks. Leaflets are distorted, reflexed, and distinctly mottled, and the youngest leaves frequently become rosetted (see Plate I, Fig. 4).

In spring-infected crops, which remain unlifted until the autumn, foliage mottling and distortion are more severe than during the early infection period. Stem necroses, as above, appear on the lower leaves which progressively wither and die. This latter symptom appears to be associated with mature plants, and has never been observed on young plants of any carrot variety. When mature plants, which had been infected for a long period, were moved to a heated glass-house during the winter, stem necroses developed rapidly on the lower leaves, and the plants died within a few weeks.

VII. MATERIALS AND METHODS

Colonies of non-infective *C. aegopodii* were maintained either on carrot or fennel (*Foeniculum vulgare* Gaertn.). The latter plant is immune to the virus, very palatable to the vector, and capable of supporting heavy aphid populations for longer periods than carrot. A semi-permanent colony was maintained on fennel plants grown in a field cage, which was shaded from all but the morning sun. This colony survived two summers.

The aphids bred on fennel were fed on carrot for a week or more before being transferred to infected plants. This precautionary procedure was adopted to overcome any possibility of reduced infectivity of the vector which might result from feeding on the sap of an immune plant.

Short term colonies of aphids were maintained satisfactorily beneath cylindrical, muslin or cellophane covered cages, constructed according to the details supplied by Hamilton (1930). Moisture-permeable cellophane was found to be the most suitable covering material except during the summer when it contracted and split very readily.

Field cages used for the propagation of test plants were originally covered with cotton marquisette, treated with a rot-proofing solution containing copper oleate or copper naphthenate. This material was more resistant to weathering than other cotton fabrics tested, but usually required replacement after two years' exposure. A more permanent type of field cage is covered with brass wire gauze (40 mesh, 34 gauge) and Windowlite (Hill 1941), a proprietary glass substitute consisting of galvanized fly-wire impregnated with a cellulose compound. The gabled roof, and top three feet of the walls of this cage, are covered with Windowlite, and the lower section of the walls with wire gauze. The cage is provided with an insect-trap entrance.

Test plants of most Umbelliferous species were grown satisfactorily in six-inch flower pots in which drainage was provided by a layer of wood charcoal.

The plants were raised in steam sterilized soil and the same soil type was used in all transmission experiments. The normal procedure was to sow seed in four equidistant clumps and thin each clump to a single seedling prior to inoculation.

In insect transmission experiments, aphids were caged on seedling plants by means of celluloid cylinders covered at one end with marquisette. These feeding cages were constructed from celluloid sheets rendered malleable by dipping in hot water, and welded together with amyl acetate or acetone. The marquisette tops were also welded in place with either of these solvents.

Aphids were transferred from plant to plant by means of a fine camel-hair brush. When air temperatures were low, it was difficult to induce adult forms of *C. aegopodii* to stop feeding, thus making transfers time-consuming and tedious. The aphids were rapidly activated by subjecting infested foliage to a temperature of 80-85°F. for an hour or more. Aphids incubated at this temperature overnight did not show any apparent loss of infectivity.

In order to avoid migration of aphids during the transfer process three plants in each pot were always covered with the feeding cages. Alternate plants were inoculated with infective and non-infective aphids respectively. The number of aphids introduced, and the length of the feeding periods varied according to the nature of the experiment. In host range studies twenty aphids were transferred to each plant and allowed to feed for at least forty-eight hours. The plants were then fumigated and removed to an insect free cage.

Injury to plant tissues sometimes occurred when heat-volatilized nicotine was used as a fumigating agent, and a method was devised whereby plants enclosed in the feeding cages were dusted with a commercial three per cent. nicotine dust without the necessity of removing the cages (Stubbs 1946).

VIII. TRANSMISSION

(a) Insect Transmission

The efficiency of *C. aegopodii* in transmitting the virus to carrots and other susceptible Umbelliferous hosts has been demonstrated in numerous experiments. In all experiments where a minimum of ten and a maximum of twenty viruliferous aphids have been fed on virus susceptible test plants, for periods ranging from twenty-four to forty-eight hours, 100 per cent. infection has occurred. The incubation period of the virus in carrot averaged seventeen days, and the shortest time recorded for the development of symptoms was fourteen days.

TABLE 1
EFFICIENCY OF SINGLE APHIDS IN TRANSMITTING THE CARROT VIRUS

Stage of Development of <i>C. aegopodii</i>	Plants Infected (10 inoculated) (%)
1 Alate females	90
2 Pre-alates with wing-buds	40
3 Second instar pre-alates	30
4 Viviparous apterous females	60

In one experiment, separate groups of twenty infective *C. aegopodii* were fed on carrot seedlings for 10 min., 30 min., 1 hr., 2 hr., 3 hr., and 24 hr. The plants were dusted at the end of each feeding period. All plants became infected with the exception of those exposed to aphids for the 10-min. period.

In another experiment, carrot seedlings were exposed to single infective aphids in the various stages of development listed in Table 1.

(b) *Insects which Failed to Transmit the Virus*

Failure to transmit the carrot virus with the Jassids *Thamnotettix argentata* Evans, *Erythroneura ix* Myers, and *Empoasca* sp., was reported earlier in this paper.

Aphids other than *C. aegopodii* which commonly infest, and are capable of breeding on, carrots include *Anuraphis tulipae* Boyer, *Hyadaphis foeniculi* Pass., *Myzus persicae* Sulzer, and *Macrosiphum* spp.

None of these species was able to transmit the virus when groups of twenty were transferred from infected to healthy carrots. The technique used in these experiments (Table 2) was identical with that used in transmission experiments with *C. aegopodii*, and in most cases control transfers were carried out with this aphid.

TABLE 2
VIRUS TRANSMISSION EXPERIMENTS WITH APHID SPECIES WHICH BREED ON CARROTS

Exp. No.	Aphid Species	Source	Results*
1	<i>Hyadaphis foeniculi</i>	Mixed colonies on infected carrot	0/8
	<i>C. aegopodii</i>		8/8
2	<i>Anuraphis tulipae</i>	Mixed colonies on infected carrot	0/4
	<i>Hyadaphis foeniculi</i>		0/4
	<i>C. aegopodii</i>		4/4
3	<i>Macrosiphum</i> sp. 1	Mixed colonies on infected carrot	0/4
	<i>Macrosiphum</i> sp. 2		0/4
	<i>C. aegopodii</i>		4/4
4	<i>Anuraphis tulipae</i>	Infected carrot	0/8
		Healthy carrot	0/8
5	<i>Myzus persicae</i>	Infected carrot	0/10
		Healthy carrot	0/10

* The numerator refers to the number of plants infected and the denominator to the number inoculated.

(c) *Graft Transmission*

In this experiment the foliage of a number of semi-mature healthy carrots was removed by transverse cuts at the bases of the petioles. Holes were then bored in each root by means of a sterile cork borer inserted at right angles to the long axis and passed completely through the central tissues to the opposite side of the root. Cores obtained in a similar fashion from infected roots were then inserted aseptically into the healthy roots, using the method described by

Murphy and M'Kay (1926). The surfaces of the grafts were sealed with melted microcrystalline paraffin wax. Controls consisted of healthy ungrafted roots and roots grafted with plugs from healthy carrots.

After completion of the grafts the roots were partly buried in trays of moist sand and stored in the saturated atmosphere of a glass-house humidity chamber for a period of four days. The average temperature for this period was 72°F., with a minimum of 60°F. and a maximum of 90°F. The roots were then planted in pots of sterile soil. Each pot contained a root grafted with an infected plug and either a healthy grafted or ungrafted root. The pots were retained in the humidity chamber for a further period of twenty-seven days and were then transferred to a field cage, as glass-house temperatures were too high for optimum symptom development. Eight days after their removal to the field cage, virus symptoms were observed on roots grafted with infected plugs. The plants were kept under observation for several weeks and, during this period, the foliage of the infected carrots became very dwarfed and intensely mottled.

At the conclusion of the experiment the roots of all the grafted plants were sectioned longitudinally and the graft unions examined. A high percentage of the grafts had knitted completely with the surrounding tissues (see Plate 3, Fig. 1), but graft unions had not occurred in those plants which failed to develop symptoms.

The results of the experiment were as follows:

Infected core grafted to healthy root	. . .	14 infected of 18 grafted
Healthy core grafted to healthy root	. . .	9 healthy of 9 grafted
Ungrafted healthy roots (18)	18 healthy

(d) Failure to Transmit the Virus by Mechanical Methods of Inoculation

Negative results were obtained in all experiments where the leaves of healthy carrot seedlings, and several non-Umbelliferous virus indicator plants, were rubbed, with and without abrasive (aloxite, 600 mesh), with undiluted sap extracted from the leaves or roots of infected carrot plants. An extract from large numbers of infective aphids also failed to infect carrot.

The addition of a reducing agent, sodium sulphite (Bald and Samuel 1934), to the inoculum, did not induce symptom development in carrot, nor did infection occur when inocula, prepared from leaves of infected carrot and slender celery extracted with dipotassium phosphate and 0.1M phosphate buffers at various pH levels, were used (Thornberry 1935; Stanley 1936). The details of these experiments are recorded in Table 3. In Experiment 5 (Table 3) inocula were prepared by grinding 2 g. samples of leaf tissue with 10 ml. aliquots of buffer solution.

(e) Failure to Transmit the Virus through Carrot Seed

On three separate occasions, a number of samples of seed gathered from infected and healthy carrots was planted in short rows in a field cage. There were no apparent differences in vigour between plants grown from the various samples, and virus symptoms did not appear.

TABLE 3
SAP INOCULATION EXPERIMENTS

Exp. No.	Source and Type of Inoculum	Method of Inoculation	Variety Inoculated	Results*	
1	Undiluted sap from leaves of naturally infected carrots	Leaf rubbing with- out abrasive	Danvers Nantes Morse's Bunching Imperator Oxheart Chantenay	0/6 0/6 0/7 0/11 0/8 0/8	
				Inoculum A	Inoculum B
2	Sap from leaves of naturally infected carrots. Inoculum A diluted 1/1 with 0.1% solution of anhydrous Na ₂ SO ₃ . Inoculum B undiluted sap	Leaf rubbing with abrasive	Chantenay Danvers Nantes Morse's Bunching Imperator Oxheart	0/50 0/5 0/7 0/6 0/8 0/6	0/50 0/5 0/7 0/6 0/8 0/6
3	Sap from leaves of naturally infected immature carrots	Leaf rubbing with abrasive	Chantenay Oxheart Nantes Imperator Danvers Morse's Bunching <i>Datura stramonium</i> <i>Nicotiana tabacum</i> <i>Solanum nigrum</i> Tomato <i>Vigna sinensis</i> <i>Phaseolus vulgaris</i>	0/70 0/5 0/5 0/6 0/5 0/5 0/6 0/6 0/6 0/4 0/6 0/6	
4(a)	Sap from roots of infected carrots	Leaf rubbing with abrasive	Chantenay	0/8	
4(b)	Water extract from infective <i>C. aegopodii</i>	Leaf rubbing with abrasive	Chantenay	0/10	
5(a)	Sap from leaves of infected carrot extracted with 3% by wt. K ₂ HPO ₄ . Reaction adjusted to pH 7.1 with 0.1M phosphate buffer at pH 7.0	Leaf rubbing with abrasive	Chantenay	0/20	
5(b)	Sap from leaves of infected carrot extracted with 0.1M phosphate buffer at pH 7.0. Reaction of buffered extract pH 6.7	Leaf rubbing with abrasive	Chantenay	0/20	

TABLE 3 (continued)
SAP INOCULATION EXPERIMENTS

Exp. No.	Source and Type of Inoculum	Method of Inoculation	Variety Inoculated	Results*
5(c)	Sap from leaves of infected carrot extracted with 0.1M phosphate buffer at pH 8.0. Reaction of buffered extract pH 7.3	Leaf rubbing with abrasive	Chantenay	0/20
5(d)	Sap from leaves of infected carrot extracted with distilled water. Reaction of extract pH 6.3	Leaf rubbing with abrasive	Chantenay	0/20
5(e)	Sap from leaves of healthy carrot extracted with distilled water. Reaction of extract pH 6.4	Leaf rubbing with abrasive	Chantenay	0/20
5(f)	Sap from leaves of infected slender celery extracted with 3% by wt. K_2HPO_4 . Reaction adjusted to pH 7.2 with 0.1M phosphate buffer at pH 7.0	Leaf rubbing with abrasive	Chantenay	0/20
5(g)	Sap from leaves of infected slender celery extracted with 0.1M phosphate buffer at pH 8. Reaction of buffered extract pH 7.5	Leaf rubbing with abrasive	Chantenay	0/20
5(h)	Sap from leaves of healthy slender celery extracted with distilled water. Reaction of extract pH 6.7	Leaf rubbing with abrasive	Chantenay	0/20

* The numerator refers to the number of plants infected and the denominator to the number inoculated.

The writer has been unable to find evidence of seed-transmission in field crops, in spite of the fact that most of the carrot seed used in Victoria during the later stages of the war was produced from virus infected seed crops. Many of the root crops inspected were grown during the period when the vector was absent, and under conditions where virus infected plants would have been detected very readily.

IX. HOST RANGE

(a) *Natural Host Plants*

The cultivated carrot appears to be the only economically important Umbelliferous host of the virus in Victoria, as disease symptoms have never been

observed on celery, parsley, or parsnip grown in close proximity to infected carrots.

A wild strain of *D. carota*, which is widely distributed in southern Victoria, is the only naturally infected weed host yet discovered.

(b) Experimentally Infected Host Plants

The virus was successfully transferred, by means of the vector, to all available varieties of cultivated and wild carrots, and to the following additional Umbelliferous species: Slender celery (*Apium ammi* (Jacq.) Urb.); sea celery (*Apium australe* Thon.); hemlock (*Conium maculatum* L.); dill (*Anethum graveolens* L.); and coriander (*Coriandrum sativum* L.).

The following species did not develop symptoms when exposed to infective aphids: Parsnip (*Pastinaca sativa* L.); celery (*Apium graveolens* L.) var. Golden Self Blanching, White Plume; caraway (*Carum carvi* L.); fennel (*Foeniculum vulgare* Gaertn.); and parsley (*Petroselinum hortense* Hoffm.) var. Triple Curled.

It seems unlikely that any of the above plants are symptomless carriers of the virus as attempts to recover the virus from them have always been unsuccessful. However, it was demonstrated that viruliferous aphids remained infective for several days when fed on celery, parsley, and fennel before being transferred to carrot plants.

C. aegopodii fed readily on all the species tested with the exception of hemlock, which appeared to be unpalatable. Infective aphids usually fed on hemlock for a sufficient time to transmit the virus to that plant, but the virus was recovered with difficulty owing to the fact that the aphids usually left the plants after a short initial feeding period, and mortality was high when they were caged on hemlock for more than one or two days. The virus has not yet been recovered from sea celery, possibly because the virus concentration in this plant is very low.

X. SYMPTOMS ON EXPERIMENTALLY INFECTED HOST PLANTS

Slender celery.—This weed, which is native to Australia, is very susceptible to the virus. The earliest symptoms appear on the youngest leaves and consist of chlorosis and recurving of the leaflets accompanied by epinasty of the petioles. Symptoms have been observed eleven days after exposure to viruliferous aphids (see Plate 2, Fig. 2).

Following the development of primary symptoms severe stunting of growth occurs and infected plants frequently die as a result of a progressive necrosis which usually commences in the lower stem region.

Slender celery is the best differential host of the virus yet discovered, as symptoms are readily discernible and are not subject to masking.

Sea celery.—When seedlings in the second true leaf stage were exposed to viruliferous aphids a mild mottling developed in the lower leaves approximately fifty days after inoculation. These symptoms did not increase in intensity and neither foliage distortion nor growth stunting occurred.

Coriander.—Chlorosis and reddening of the foliage was accompanied by severe stunting and infected plants usually died. This plant is a recent addition to the host range of the virus, and its reaction has not yet been studied in detail.

Hemlock.—Symptom development is very rapid. The earliest symptoms, consisting of chlorotic and necrotic flecking of the lower leaves, have been observed eleven days after exposure to the vector. These symptoms become more pronounced about thirty days after inoculation. The leaflets become slightly reflexed and irregular chlorotic patches appear on the petioles, which become twisted or bent in a similar fashion to that observed in carrot. The growth rate of infected plants is slightly retarded.

Dill.—Infected plants were severely dwarfed and developed a purplish-red coloration of the lower leaves.

Wild carrot.—Wild forms of *D. carota* collected in the Dandenong Ranges, in south and east Gippsland, and in south-eastern New South Wales, were all susceptible to the virus. When grown under similar conditions to the cultivated carrot, these forms produced a rosetted type of foliage growth and white forked roots.

Foliage symptoms on seedling plants were less obvious than those on cultivated varieties, but growth stunting was equally severe. Leaflet distortion, petiole twisting, and mosaic mottling were not obvious, but a characteristic red pigmentation occurred in the petioles and margins of the lower leaves. This latter symptom was more pronounced when larger plants were infected.

XI. REACTIONS OF CULTIVATED AND WILD VARIETIES OF *DAUCUS CAROTA* TO THE VIRUS

A number of named carrot varieties, and several unnamed selections and wild strains of *D. carota*, were exposed to natural infection under field conditions. Field trials were usually sown in spring, but supplementary sowings were sometimes made in autumn. These trials were conducted over a three-year period, but not all the varieties listed in Table 4 were available for testing during this period.

The reactions of individual varieties to the virus were assessed by the degree of foliage distortion, mottling and dwarfing, and root size at the time of lifting. In these trials lack of foliage vigour was always accompanied by a reduction in root size.

All varieties tested were susceptible to infection and, as the majority of the varieties reacted in a similar fashion to the virus, it was not possible to apply any form of disease rating.

Several Australian varieties and selections exhibited marked tolerance to the virus and of these a Victorian selection showed most promise. The history of these varieties is obscure, but it is thought that they originated from selections, made by growers, from vigorous types which sometimes occur in diseased crops of susceptible varieties. The author has observed virus tolerant "strangers" in infected crops of the Chantenay variety, which were characterized by vigorous foliage and coarse Intermediate-type roots.

TABLE 4
REACTIONS OF CULTIVATED AND WILD VARIETIES OF *D. CAROTA* TO THE VIRUS

Variety	Origin of Seed	Reaction to Carrot Virus
Chantenay (4 lines)	U.S.A.	Susceptible
Chantenay	England	Susceptible
Chantenay	Tasmania	Susceptible
Champion Intermediate	W. Australia	Tolerant
Danvers	U.S.A.	Susceptible
Danvers	England	Susceptible
Early Market (2 lines)	England	Susceptible
Hutchinsons'	U.S.A.	Susceptible
Imperator (2 lines)	U.S.A.	Susceptible
Imperator X Champion Intermediate	W. Australia	Tolerant
James' Intermediate (Scarlet)	England	Susceptible
Johnson's Maincrop	England	Susceptible
Long Orange	U.S.A.	Susceptible
Morse's Bunching	U.S.A.	Susceptible
Nantes	U.S.A.	Susceptible
Nantes	Australia	Susceptible
Nantes Gribvorskaya	U.S.S.R.	Susceptible
Oxheart	U.S.A.	Susceptible
Oxheart	England	Susceptible
Paris Carotel	U.S.S.R.	Susceptible
Red-stalked Intermediate	Australia	Tolerant
St. Valery	Australia	Susceptible
Stump-rooted Intermediate	England	Susceptible
Unnamed selection	Victoria	Tolerant
Valeria	U.S.S.R.	Susceptible
Scarlet Horn (2 lines)	U.S.A.	Susceptible
<i>D. carota</i> , wild (2 strains)	India	Susceptible
<i>D. carota</i> , wild (2 strains)	Baluchistan	Susceptible
<i>D. carota</i> , wild (3 strains)	Iran	Susceptible
<i>D. carota</i> , wild (4 strains)	Victoria and N.S.W.	Susceptible

XII. THE VECTOR

(a) Distribution

C. aegopodii is widely distributed in Victoria and is a serious economic pest of carrot, celery, parsnip, and parsley.

The aphid has also been found infesting willows and sea celery growing in situations widely separated from agricultural areas.

(b) Taxonomy

The taxonomy of *Cavariella aegopodii* has been described in detail by Theobald (1927), but certain morphological features of the Victorian *Cavariella* resemble more closely those of the aphid referred to by Essig (1938) as *C. capreae* Fab. than those of *C. aegopodii*. In view of this discrepancy specimens taken from carrot and fennel in Victoria were forwarded to Essig, who expressed the opinion that they were identical with the American *C. capreae*. He agreed that there was "reason for some confusion concerning the difference

between *capreae* and *aegopodii*," but suggested adherence to the European in preference to the American terminology.

Theobald, on the other hand, separates *C. capreae* from *C. aegopodii*, but in his description of the former, makes reference to the fact that "Schouteden and some other authors place Scopoli's *Aphis aegopodii* as a synonym" of *C. capreae*.

(c) Food Plants

In Victoria, the natural host range of *C. aegopodii* approximates closely to that of *C. capreae* in the United States (Essig 1938), but is considerably wider than that described for *C. aegopodii* in England (Theobald 1927).

Naturally occurring colonies of *C. aegopodii* have been found on the following Umbelliferous species: caraway, carrot, celery, celeriac, fennel, hemlock, sea celery, parsley, parsnip, and slender celery.

The aphid completed its life-cycle on all the above plants with the exception of hemlock. All attempts to breed the vector on this plant were unsuccessful, but naturally infested hemlock plants were observed on one occasion, growing in close proximity to heavily infested carrots. Aphids in all stages of development were found concentrated on the under surfaces of the lower leaves of these plants. The reason for the failure of more recent attempts to establish colonies on hemlock is not known, but this plant is obviously unpalatable to the vector and cannot be regarded as a normal food plant.

The aphid also occurs naturally on willow, principally *Salix vitellina*. When the winter dormancy of this plant was broken by striking stem cuttings under hot-house conditions, colonies of aphids developed on the newly emerged leaves, thus suggesting that *C. aegopodii* overwinters in the egg stage on willow in Victoria. Under field conditions aphids usually appear after bud-burst in the spring and, in some districts, particularly east Gippsland, willow appears to be an important host reservoir of the aphid.

(d) Feeding Habits of the Vector and Tissues Involved

The difficulty experienced in inducing the vector to withdraw its stylets when feeding suggested that it was not a surface feeder. This belief was further strengthened by the failure to transmit the virus mechanically, this characteristic being consistent with that of many viruses whose vectors are phloem feeders.

In a histological study of the feeding habit of *C. aegopodii* microtome sections were cut of aphids feeding on carrot foliage (see Plate 3, Fig. 2) using the technique described by Dykstra and Whitaker (1938). Permanent mounts were made of preparations showing stylets which were complete either in single, or in serial sections. The sections were stained with a safranin-light green combination. The most satisfactory results were obtained when sections were stained in a 0.5 per cent. solution of safranin in 47.5 per cent. alcohol for 18-24 hours and counterstained in a 0.2 per cent. solution of light green in 95 per cent. alcohol for 2-3 minutes.

Microscopic examination of the sections revealed that the aphid invariably fed on phloem tissue and that the passage of the stylets was usually intercellular through the cortex (see Plate 3, Fig. 3). The absence of any plasmolysis indicated that feeding did not occur in cortical tissues.

(e) *Previous Records of Virus Transmission by Cavariella aegopodii*

The author has been unable to find any record of virus transmission by *C. aegopodii*, but Smith (1937) reported that Severin found *C. capreae* capable of transmitting the Cauliflower Mosaic virus, although the aphid did not breed on cauliflower. Severin and Freitag (1938) also recorded transmission of Western Celery Mosaic virus with this aphid, which bred on celery.

In view of the fact that Essig, as reported above, considers the American *C. capreae* to be identical with the Victorian *C. aegopodii*, there appears to be no justification for claiming a first record of virus transmission for *C. aegopodii*.

XIII. THE VIRUS IN RELATION TO THE VECTOR

The classification and identification of virus diseases is based largely on symptomatology, host range, virus properties *in vitro*, and virus-vector relationships.

The physical properties of the carrot virus could not be determined because of the failure to effect its mechanical transmission, and it therefore became necessary to study its relationship with the vector.

Insect-transmitted viruses have been classified according to their persistence or non-persistence within their vectors (Bawden 1943), and it has been claimed that some viruses of the former type undergo an "incubation" or "latent period" before their vectors develop infective ability.

It was found that the carrot virus persisted in its vector for long periods, but further experiments were initiated to determine firstly, the length of time the vector remained infective after a known feeding period on an infected plant, and secondly, the existence or otherwise of a latent period.

In all of these experiments non-infective apterous females were transferred, after an initial period of starvation, to single detached leaves of infected Chantenay carrots for a known infection feeding period (IFP). Groups of ten, selected from aphids which were feeding, were then serially transferred at regular intervals to healthy plants, for varying test feeding periods (TFP). Carrot was used initially as the test plant, but was eventually replaced by slender celery, which proved more satisfactory because of its greater susceptibility to the virus.

Early experiments indicated that a latent period occurred, but these results conflicted with those of more recent experiments in which the vector, after infection feeds of 24, 18, and 3 hr., and test feeds of 24, 3, and 1 hr. respectively, transmitted the virus to the first series of healthy transfer plants in each experiment.

The earlier results (which are not recorded) are regarded as being unreliable, and may be attributable to a faulty technique or to a low virus concentration in the plants on which the aphids were fed.

The virus persisted in the vector for long periods when the infection feed was of long duration, and for shorter periods when the infection feeding time was decreased, and higher percentages of test plants became infected as the infection or test feeding times were lengthened. These results, which are recorded

in Table 5, are in agreement with those obtained by Watson (1940), in transmission studies with *Myzus persicae* and the Sugar-Beet Yellows virus.

TABLE 5
PERSISTENCE OF THE CARROT VIRUS IN CAVARIELLA AEGOPODII

Exp. No.	Test Plant	No. of Replicates	IFP (hr.)	TFP (hr.)	No. of Transfers	No. of Transfers in which Infection Occurred	No. of Days Vector remained Infective
1	Slender celery	3	48	24	18	18	18
2	Slender celery	4	24	24	7	7	7
3	Slender celery	4	18	3	7	7	4
4	Slender celery	3	3	1	8	7	2
	Carrot	3	3	1	8	6	2

XIV. EFFECT OF VIRUS INFECTION ON CARROT SEED PRODUCTION

(a) Incidence of Root-Rot in Infected and Healthy Seed Plants

Experiment 1.—A preliminary experiment, designed to elucidate a suspected relationship between virus infection and rotting of carrot roots transplanted for seed, was commenced in June 1944.

Infected and healthy roots of the Chantenay variety were selected from a summer sown crop in a Werribee market-garden. Although the vector was present, only small patches of the crop were infested, and less than 20 per cent. of the plants exhibited foliage mottling. The roots of infected plants were not noticeably smaller than those of apparently healthy plants, indicating that infection was of recent origin. It is possible that some of the supposedly healthy plants selected were incipiently infected as their health status could only be assessed by the presence or absence of foliage mottling. Roots were topped immediately after lifting and dipped in a nicotine solution to destroy any aphids which might be present.

Equal numbers of virus infected and healthy roots of uniform size, free from blemishes and growth cracks, were then planted in single row plots in a recently cultivated pasture area at Burnley. Each plot consisted of thirty-six roots planted equidistantly, and five plots of infected roots were alternated with five healthy plots.

During the winter, foliage growth was very slow and the plants remained free from aphids until September. Unfortunately, at the end of this month the plants became heavily infested with pea mite (*Halotydeus destructor* Tucker), which could not be satisfactorily controlled by frequent applications of nicotine dust or sprays. The experiment was discontinued on October 10, 1944, when it became apparent that the less vigorous foliage of the virus infected plants was being affected more severely by the pea mites than the foliage of the healthy plants.

The results recorded in Table 6 are the summations of weekly counts of completely rotted roots; counting commenced on September 4 and concluded on October 10. These results, although incomplete, indicated that virus infection was a predisposing factor for the rotting of seed-roots.

TABLE 6
INCIDENCE OF ROOT DECAY IN HEALTHY AND VIRUS INFECTED SEED PLANTS

Plot No.	No. Roots Rotted of 36 Planted		Rotted	
	Healthy	Infected	Healthy (%)	Infected (%)
1	1	7	2.8	19.4
2	1	9	2.8	25.0
3	0	5	0	13.9
4	0	6	0	16.7
5	0	7	0	19.4
Mean	0.4	6.8	1.1	18.8

Experiment 2.—This experiment was designed to eliminate many of the variants which unavoidably occurred in Experiment 1. Unfortunately it was necessary to compromise between transplanting roots at a time most suitable for seed production purposes when healthy control plots would most certainly become infected after establishment, or transplanting the roots at a less favourable time and covering them with cages until the vector disappeared in early summer. In order to attain the main objective of the experiment the latter procedure was adopted.

(i) *Method.*—On March 9, 1945, seed of the Chantenay variety was sown in each of eight three-rowed plots, in a uniform sandy loam soil at Burnley which had been treated, prior to sowing, with a complete fertilizer mixture applied at a rate equivalent to 9 cwt. per acre. These plots were arranged in two rows and were separated by paths two feet wide along their long and short axes. A pair of plots, one in each row, constituted a block. Each plot was completely covered with a muslin cage 4 ft. long, 3 ft. wide, and 18 in. high. These cages were provided with arm-holes to enable weeding, cultivating, and thinning to be performed without risk of infection from winged aphids (see Plate 4, Fig. 1).

On September 24, 1945, groups of infective *C. aegopodii* were introduced to single cages selected at random in each of the four blocks, and non-infective aphids to the four remaining cages. During October the vector multiplied rapidly and all plots became heavily infested. The plants exposed to viruliferous aphids developed typical virus symptoms and their foliage became stunted. The control plants supported a heavier infestation than the virus infected plants, without any apparent loss of vigour.

On November 8, 1945, the cages were sprayed thoroughly with a nicotine sulphate solution (1 in 400) and, on the following day, the roots were pulled, topped, and dipped in nicotine solution to destroy any aphids which might have survived the spraying.

Twelve evenly matched roots, free from growth cracks and blemishes, were then selected from one plot and used as a standard for selection from the remaining plots. The total weight of the standard group was 790 g. and, in order to qualify, each group of uniformly sized roots was required to be within 10 g. of this weight. Two hours after dipping, these groups, comprising individual replicates, were each planted in double-rowed plots in an adjacent area, and in positions corresponding to their locations in the original randomization. The roots were planted vertically every 12 in., in rows 24 in. apart, and covered to the bases of the petioles with compacted soil. Each root was then enclosed in a cylindrical muslin-covered cage.

A subsidiary experiment was also commenced in an insect-free field cage. In this experiment an infected and a healthy root were planted in each of 28 porcelain pots containing acid washed sand. The pots were watered twice weekly with 250 ml. of a complete nutrient solution suitable for the growth of carrots.

The roots established in the field plot experienced ideal conditions during the ten days following transplanting, owing to the occurrence of heavy rain and a period of cool weather.

(ii) *Growth Rate*.—During the following month the roots in the field plot produced considerable foliage growth. The cages were removed on December 13, 1945, and the plants were sprayed with a proprietary DDT emulsion every few days until, with the occurrence of normal summer temperatures, the danger of winged aphids passed.

On December 15, 1945, the height of every plant in the field plot was measured. As the plants had not formed seed stalks at that stage, measurements were obtained by bunching the leaves together in one hand, whilst recording the height of the tallest leaves to the nearest inch. A statistical analysis of these measurements (see Table 7), showed that initial differences in vigour, which were highly significant, occurred between the healthy and virus infected seed plants. Growth differences were even more apparent between the healthy and infected roots grown in sand culture, but as a number of plants in this trial had produced seed stalks, foliage measurements could not be obtained.

(iii) *Incidence of Root-Rot*.—During the period January to April 1946, the foliage of a number of the virus infected plants, in both experiments, wilted and died. Examination of the roots of these plants showed that a complete destruction of the cortical tissues had occurred, and in most cases only a shrivelled vascular cylinder remained. The numbers of plants which died in this manner before producing seed are recorded in Table 7.

TABLE 7
COMPARATIVE GROWTH AND SURVIVAL OF HEALTHY AND VIRUS INFECTED SEED PLANTS

	Foliage Height in Field Plot on 13.xii.45 (in.)	Mortality from Root Decay	
		Field Experiment (%)	Sand Culture Experiment (%)
Virus infected	6.3	37.5	64
Healthy	13.9	0	0

At the conclusion of the experiment, roots of all plants were individually examined. It was found that healthy roots had considerably increased in girth since transplanting, and had produced sizeable lateral roots, some of which had attained a diameter of one centimetre. The surviving infected roots presented a marked contrast, as they had not increased in girth and their lateral roots were of a fibrous nature.

(b) *Effect of Virus Infection on Seed Yield*

Throughout the summer, autumn, and early winter, the field plot established in Experiment 2 was sprayed at weekly intervals with DDT emulsion, to combat Rutherglen bug (*Nysius vinitor*) and *H. foeniculi*, both serious pests of carrot seed crops in Victoria. Seed umbels were harvested as they matured and stored in paper bags in the laboratory.

Surviving plants in the infected plots were very stunted and chlorotic and the most severely affected plants only produced primary umbels. The healthy plots, on the other hand, were uniformly vigorous and all plants developed many secondary umbels in addition to primary umbels (see Plate 4, Fig. 2).

(i) *Seed-Cleaning Method*.—Umbels harvested individually from each replicate were air-dried for several months, and then hand stripped. These bulk samples were freed from awns, and infertile seed was largely destroyed by rubbing between a rubber-covered wooden block and a corrugated rubber mat. The resultant debris was removed by means of three British Standard sieves (8 mesh, 16 mesh, and 20 mesh to the inch, respectively) and the draught from an electric fan. The three sieves were placed together in the order mentioned and held immediately above a fan, the blades of which were inclined at a slight angle to the horizontal. Seed was then poured into the topmost sieve and agitated for several minutes. The coarse top sieve allowed the passage of all the seed, but removed most of the coarse stalk material; the middle sieve held back the largest seed, or seed from which awns had not been completely removed; the bottom sieve prevented the passage of the smallest seed and most of the fine debris was blown away by the draught from the fan. The contents of the middle sieve were then transferred to the fine mesh sieve, and carefully agitated above the fan. This final operation removed most of the remaining debris, including light infertile seed which had survived the rubbing process, and the resultant sample compared favourably in appearance with good commercial samples.

(ii) *Germination Tests and Seed Yield*.—Laboratory germination tests were conducted in triplicate with samples of 100 seeds from each replicate. The germination of most samples was unexpectedly low, and seed from infected plots had a higher test than seed from healthy plots. Seeds which failed to germinate usually produced a copious fungal growth which was identified (following pathogenicity tests) as *Macrosporium carotae*, Ell. & Langl. This fungus is of minor importance on carrots in Victoria, but has been observed attacking and partially defoliating mature crops of susceptible varieties grown under wet conditions.

The lower infection occurring in seed from virus infected plots would appear to be due to the fact that most of this seed was produced in primary umbels, which matured earlier than secondary umbels, and when the fungus was less active. The relatively high percentage of infected seed from control plots was rather surprising, as the foliage of the seed plants showed only slight infection during the growing season.

Germination of the seed was improved after treatment with a fungicidal dust containing 1 per cent. ethyl mercury phosphate (New Improved Semesan Jr.), but the fungus was not completely controlled by this treatment.

The results of the germination tests together with the mean yields of cleaned seed from healthy and virus infected plots are recorded in Table 8.

The differences in seed yield from healthy and infected plots were highly significant.

TABLE 8
COMPARATIVE SEED YIELDS OF HEALTHY AND VIRUS INFECTED CARROTS

	Yield of Cleaned Seed (Mean of 4 Replicates)	Mean Germination	
		Untreated Seed	Treated N.I. Semesan Jr.
	(g.)	(%)	(%)
Healthy	124.5	36.5	51.0
Virus infected	16.7	44.0	58.0

(c) *Factors Associated with Root Decay*

While the above experiment was in progress an attempt was made to elucidate the factors responsible for root decay in seed plants.

The author was unable to find any record of a similar problem in the virus literature, but investigations by Kristoffersen (1921) concerned with the resistance of carrot varieties to winter storage rots, suggested a promising line of approach to the problem. Kristoffersen claimed that a positive correlation existed between low invert sugar content and rot resistance of stored roots, and that the correlation was more marked if the relative percentage of invert sugar to total sugar was considered, instead of the total percentage of invert sugar. A relative percentage of invert sugar to total sugar in excess of 45-50 per cent. indicated that the roots would rot in winter storage and it was also suggested that invert sugar might provide a better medium than cane sugar for the growth of parasites associated with root decay.

(i) *Root Analyses.*—In pursuance of this line of investigation samples of roots were selected for analysis from each of the muslin covered plots in Experiment 2. These samples were selected at random when the plots were harvested, and each replicate was analysed for reducing sugar and non-reducing sugars.

The results of the analyses are summarized in Table 9.

The above analytical determinations showed that the invert sugar content of virus infected roots was lower than that of healthy roots and that Kristoffersen's hypothesis did not explain the susceptibility of the former to root decay.

TABLE 9
SUGAR CONTENT OF INFECTED AND HEALTHY CHANTENAY CARROT ROOTS

Sample	Reducing Sugars (as Invert)			Non-Reducing Sugars (as Sucrose)		
	As Received	On Dry Basis		As Received	On Dry Basis	
		(%)	(Angle)		(%)	(Angle)*
Healthy roots	3.88	38.4	38.28°	1.23	11.9	20.16°
Infected roots	3.50	32.5	34.69°	1.72	16.1	23.64°
Difference for significance 1% level			3.38°			—
Difference for significance 5% level			—			2.34°

* Percentage figures have been transformed to angles (= arcsine $\sqrt{\text{percentage}}$) as described by Snedecor 1940, and examined statistically by the analysis of variance method (Fisher and Wishart 1930).

Similarly, the relative percentage of invert sugar to total sugar was lower for infected roots (67 per cent.) than for healthy roots (76 per cent.) and, according to Kristoffersen, both would have rotted in winter storage, the latter being most susceptible. Although storage tests were not conducted, it is considered that such an assumption would have been illogical, in view of the performance of healthy and infected roots in the seed-production trial.

(ii) *Root Inoculations with Fungal Organisms.*—In order to determine whether virus infected carrot roots provided a better medium for fungal growth than healthy roots, inoculation experiments were conducted with organisms found associated with root decay in carrot seed crops. The following organisms were used: *Alternaria radicina* (M., Dr., & E.); *Sclerotium* sp.; *Fusarium* spp. (4); and *Verticillium* sp.

Needle prick inoculations with each organism were replicated three times on separate infected and healthy roots, which were then suspended in pairs over a free water surface in glass refrigerator jars, and incubated at 25°C.

Observations over a period of thirty days showed that root decay produced by the various organisms proceeded at a similar rate on both infected and healthy roots. The organisms were all weak parasites, or saprophytes, with the exception of *A. radicina* and *Sclerotium* sp., neither of which was associated with Victorian seed-crop failures. The species of *Fusaria* tested were the commonest organisms isolated from roots in early stages of decay during the investigation.

These results suggest that undue significance has been attached to the apparent biological breakdown of roots in carrot seed crops and that this phenomenon is a secondary development not responsible for the death of seed plants. It is postulated that the breakdown of infected transplanted carrot roots is a form of necrotic collapse resulting from virus infection, which may be accelerated by transplanting, but which does occur in plants which have not been transplanted.

The factors associated with the development of this phase of the disease are imperfectly understood, but it is believed that necrotic symptoms do not appear in carrot roots until plants are approaching maturity. All cultivated varieties of *D. carota* vary considerably, and it is thought that individual plants in a susceptible variety vary in their reactions to the virus, thus accounting for the fact that some seed plants do not develop root decay. In more susceptible species, like slender celery and coriander, necrotic symptoms usually occur, and very few plants survive the disease.

Environmental conditions also appear to be of importance in determining the mortality rate in seed crops grown from infected roots. The incidence of root-rot was higher in the above sand culture experiment than in the field trial (Experiment 2), yet the roots were obtained from the same source and lifted at the same time. In the former experiment, however, the plants were subjected to higher temperatures in a field cage, and grown in sand with low water-holding capacity. In seed crops dependent upon natural rainfall, the poorly developed lateral root systems of infected plants would be unable to supply their water requirements during dry periods, or to withstand the considerable leverage imposed by foliage movements in windy weather.

XV. CONTROL

(a) Regulation of Time of Sowing

Virus free carrots can be produced in Victorian localities where natural rainfall or irrigation facilities permit the establishment of summer sown crops. In some districts late November sowings will remain healthy, whereas, in others, carrots should not be sown before mid-December.

Rigid sowing dates cannot be recommended for any one locality as the occurrence of late spring rains in an abnormal season may prolong the aphid infestation well into December, or even until early January. However, if such conditions do occur, aphid control measures should be regularly applied until the advent of normal summer temperatures prevent further multiplication of the vector.

(b) Virus Control by Combating the Vector

The few successful attempts "to control virus diseases in the field by combating the vectors" are summarized by Bawden (1943), who also suggests that such measures may be practical in small scale intensively cultivated crops. All successful vector control experiments have depended upon regular applications of insecticidal sprays or dusts during the period when the vectors were active. In preliminary experiments conducted during 1943 at a Dandenong market-garden, nicotine sprays and dusts applied to spring sown carrots at weekly intervals failed to control the disease.

During 1945-46 a further experiment was conducted at Burnley to test the efficacy of the then new insecticides DDT (dichlordiphenyltrichlorethane) and 666 (hexachlorocyclohexane) in controlling *C. aegopodii*.

(i) *Method*.—On September 25, 1945, an experimental area was sown with carrots (var. Chantenay) in rows one foot apart.

The following spray treatments, which were applied at approximately weekly intervals, were replicated four times in randomized blocks. Plot units consisted of four parallel rows fifteen feet long.

DDT. An aqueous emulsion containing 0.1 per cent. DDT in aromatic solvent (prepared from an emulsion concentrate containing 20 per cent. para para isomer of DDT).*

666. An aqueous emulsion containing 0.1 per cent. hexachlorcyclohexane in aromatic solvent (prepared from an emulsion concentrate containing 1.9 per cent. of gamma isomer of hexachlorcyclohexane).*

Nicotine sulphate (40 per cent. nicotine) 1/600 + white oil emulsion 1/150.

Control plots were not sprayed.

The above insecticides were applied by means of a knapsack, hand operated spray pump. Spraying was commenced on October 12, 1945, and concluded on December 15, 1945—a total of eight applications.

At the appropriate time the central rows of each plot were thinned to fifty plants per row. The outer buffer rows were not thinned with the same degree of accuracy.

The experimental plot received normal culture treatment and was watered by overhead sprinklers. Watering was not required during the spraying period.

(ii) *Aphid Control*.—Observations on November 9, 1945, showed that the control plots were heavily infested with *C. aegopodii*; a few aphids were present on 666 and nicotine sulphate-white oil plots, but aphids could not be found on DDT plots. A heavy infestation persisted on the unsprayed plots until the occurrence of high temperature conditions in mid-December.

On the day preceding the final spray application aphid counts were obtained on samples of twenty-five leaflets selected at random from the central rows of each replicate.** The results of these counts, which are recorded in Table 10, confirmed the earlier observations that DDT was more effective than 666 and nicotine sulphate-white oil in controlling *C. aegopodii*.

TABLE 10
POPULATIONS OF *C. AEGOPODII* ON SPRAYED AND UNSPRAYED CARROT FOLIAGE

Treatment	Mean Numbers of Aphids on Samples of 25 Leaflets	
	Alate	Apterae
DDT	1.25	0
666	0.5	2
Nicotine sulphate	3.75	4
Control	13.0	1469

At an early stage in the experiment, differences in foliage vigour between the sprayed and unsprayed plots became apparent, and by mid-November the DDT replicates were more vigorous than those of the other treatments and had produced foliage at least twice the height of that of the unsprayed plots. Foliage

* Products of Imperial Chemical Industries of Aust. & N.Z. Ltd.

** Aphid counts were made by Mr. T. W. Hogan, Senior Entomologist, Department of Agriculture, Victoria.

dwarfing in the control plots resulted from the combined effect of virus infection and aphid feeding injury, but the former was apparently most important, as only slight recovery occurred when the aphids disappeared.

(iii) *Virus Control*.—On February 25, 1946, the numbers of virus infected plants in the central rows of each replicate were recorded, and the results (see Table 11) subjected to an analysis of variance.

TABLE 11
EFFECT OF SPRAY TREATMENT ON VIRUS INCIDENCE

Treatment	Virus Infected Plants on 25.ii.46	
	(%)	(Angle)
DDT	68.2	55.97°
666	86.5	70.07°
Nicotine sulphate	88.7	71.06°
Control	100	90.00°
Difference for significance 1% level		18.95°
Difference for significance 5% level		13.18°

It is of interest to note that the order of effectiveness of the three insecticides was accurately forecast by the vector population counts recorded in Table 10.

(iv) *Yield*.—On April 9, 1946, roots from the centre rows of each plot were pulled, graded, and weighed. Grading was carried out according to the following standards which were adopted for carrots grown under contract to the Commonwealth during the last war: "To be sound, clean, of minimum diameter 1½ in. and maximum 4 in., with a minimum length of 4 in. when the diameter is between 1½ in. and 2 in. . . . fresh (not withered), reasonably free from growth cracks, excessive rootlets and woodiness, and free from forking and all damage including damage resulting from disease and insect pests. Mis-shapen or malformed roots, or roots producing seed heads prior to harvesting not to be included." The results of this experiment, which were subjected to an analysis of variance, are recorded in Table 12.

TABLE 12
EFFECT OF SPRAY TREATMENTS ON YIELD OF CARROTS

Treatment	Total Wt. (lb.)	Marketable Wt. (lb.)	Unmarketable Wt. (lb.)		
			Undersized	Split	Rotted
DDT	17.7	8.6	2.9	6.2	2.4
666	14.3	5.2	4.4	4.7	2.2
Nicotine sulphate	12.8	4.6	3.6	4.6	2.4
Control	9.0	1.3	4.3	3.5	0.8
Difference for					
significance 1% level	7.4	6.0	—	—	—
Difference for					
significance 5% level	5.1	4.2	—	—	—

In comparing the results obtained from the various treatments with the control, DDT alone produced highly significant increases in total yield and yield of marketable roots, the latter being more than a sixfold increase. The total yield of roots was also significantly increased (5 per cent. level) by 666.

The amount of virus infection in the DDT and 666 plots was significantly less (1 per cent. level) than in the untreated plots and disease reduction was significantly greater (5 per cent. level) from DDT.

While the Burnley experiment was in progress, Taylor (1945) reported the results of spraying trials against *C. aegopodii* on carrots in New Zealand.* In these trials an aqueous suspension of DDT gave less effective control than 666 and nicotine sulphate, but the results of the two experiments are not comparable, as a soluble form of DDT was used in the Burnley experiment.

In a recent report from the United States, Pound and Chapman (1947) recorded control of the Aster Yellows virus in carrots by controlling its vector, *Macrostes divisus*. In this experiment six applications of a wettable DDT spray, at ten day intervals, increased the yield of carrots by four tons above the unsprayed control and decreased virus infection by 38 per cent.

The author has been unable to find any further records of the control of virus diseases by means of DDT, but it is anticipated that practical field control of other insect transmitted viruses will be demonstrated in the future, as the residual effect of this insecticide overcomes one of the greatest disadvantages of older insecticides which have been used unsuccessfully in many vector control experiments.

(c) *Disease Control by the use of Virus Tolerant Varieties*

In the course of field varietal trials the existence of degrees of "resistance" became apparent between the various virus tolerant varieties and, in order to evaluate the resistance of the most promising varieties, a yield trial was sown at Burnley on October 4, 1945.

(i) *Method.*—In this trial, a commercial line of seed of the susceptible Chantenay variety was chosen as a standard for comparison with the following virus tolerant selections:

- (1) Champion Intermediate (syn. Osborne Park), from Balcatta, Western Australia.
- (2) Emperor X (stated to be the result of a cross between Emperor and Champion Intermediate), from Balcatta, Western Australia.
- (3) Victorian strain (selected, at Burnley, from a virus tolerant strain of unknown origin, obtained from a metropolitan market-garden in 1942).

Each of the above varieties, and Chantenay, was replicated four times in randomized blocks. Individual plots consisted of six rows spaced one foot apart by twenty-five feet long, the two outer rows being regarded as buffers.

At thinning time a total of 150 evenly spaced plants was left in each plot, excluding the two buffer rows.

* The carrot virus disease has not been recorded in New Zealand.

All varieties became heavily infested with the vector shortly after emergence and 100 per cent. virus infection occurred. Aphid control measures were not applied.

The experiment was harvested on April 11 and 12, 1946, and the roots from individual plots were weighed immediately after lifting. The roots were graded as in the above vector control spraying experiment.

(ii) *Yield*.—The yield data obtained from the experiment were examined statistically. The relevant results are summarized in Table 13.

TABLE 13
COMPARATIVE YIELDS OF VIRUS TOLERANT AND VIRUS SUSCEPTIBLE CARROT VARIETIES

Variety	Total Yield	Yield Marketable Roots			Red Core (%)
	(lb.)	(lb.)	(%)	(Angle)	
Victorian strain	43.9	30.7	61.2	51.49°	17
Champion Intermediate	35.0	17.2	37.5	37.73°	52
Imperator X	34.6	14.4	28.3	31.98°	41
Chantenay	21.3	8.2	21.2	27.21°	96
Difference for significance 1% level	11.4	6.2		6.44°	
Difference for significance 5% level	7.9				

In the above experiment it was unfortunate that a comparison could not be made between the yields of the four varieties in the absence of virus infection. However, the results showed that the Victorian strain which exhibited least foliage mottling and dwarfing also produced the highest yield and percentage of marketable roots, whereas Chantenay, the most severely affected variety, occupied the converse position. Moreover, Chantenay is generally regarded as being a high yielding variety, and it is, therefore, reasonable to assume that the yield differences were largely attributable to the virus.

Roots produced by the Victorian strain were of a fairly uniform type resembling, but slightly shorter and more stumped than, those of the Champion Intermediate variety. They were somewhat paler than those of the other varieties and were predominantly yellow cored. The roots pulled "cleanly" and lacked the unsightly lateral root depressions sometimes shown by the Champion Intermediate variety.

A hollow crown was more characteristic of this strain than of Champion Intermediate, and the junction of the petioles with the crown in the former, resembled that of quality varieties like Chantenay. The Champion Intermediate variety is prone to "neckiness" and "greening" in the crown region. Reference to Plate 4, Figure 3, will show some of the features which distinguish the two varieties.

In June 1947, approximately 10 cwt. of roots grown from the Burnley selected strain were planted, after further selection, at the Horticultural Research Station, Scoresby. The seed obtained from this crop will serve as a nucleus for

further agronomic improvement of the variety. The variety will ultimately be named and distributed to the seed trade.

XVI. DISCUSSION

Certain symptoms of the Australian carrot disease resemble those described for the American diseases caused by the Californian Aster Yellows (Severin 1932) and Western Celery Mosaic (Severin and Freitag 1938) viruses, but there seems to be little doubt that the local virus is distinct from either of these viruses.

The virus resembles Aster Yellows in being a persistent virus, but differs in its mode of transmission, and in its host range. It is unlikely, moreover, that the presence in Australia of Aster Yellows would have remained unnoticed, because it causes economically serious diseases in a number of completely unrelated host plants.

There appears to be little or no resemblance between the carrot virus and the Western Celery Mosaic virus. The latter, as the name suggests, infects celery, which is immune to the carrot virus, is mechanically transmissible, and is non-persistent in a number of aphid vectors which feed on celery.

The Australian disease appears to be unique in that it has a specific vector which is itself a serious pest of carrots, and this aphid occurs in very large numbers when environmental conditions are favourable, thus carrying the virus to every plant in infested crops.

XVII. ACKNOWLEDGMENTS

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The analytical determinations were conducted by Mr. W. R. Jewell, Agricultural Research Chemist, Department of Agriculture, Victoria, and weed hosts were identified by Mr. A. W. Jessep, Government Botanist, National Herbarium, Melbourne.

XVIII. REFERENCES

- BALD, J. G., and SAMUEL, G. (1934).—Some factors affecting the inactivation rate of the virus of tomato spotted wilt. *Ann. Appl. Biol.* **21**: 179-90.
- BAWDEN, F. C. (1943).—"Plant Viruses and Virus Diseases." 2nd Ed. (Chronica Botanica Co.: Massachusetts.)
- DYKSTRA, T. P., and WHITAKER, W. C. (1938).—Experiments on the transmission of potato viruses by vectors. *J. Agric. Res.* **57**: 319-34.
- ESSIG, E. O. (1938).—Aphids feeding on celery in California. *Hilgardia* **11**: 459-92.



Fig. 1



Fig. 2



Fig. 3



Fig. 4



Fig. 1



Fig. 2



Fig. 1



Fig. 2

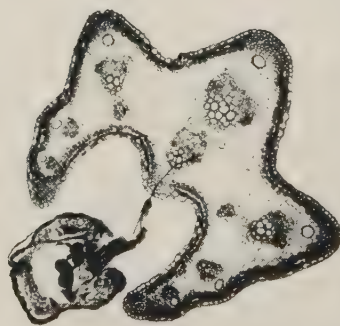


Fig. 3



Fig. 1



Fig. 2



Fig. 3

- FISHER, R. A., and WISHART, J. (1930).—Some difficulties in the statistical analysis of replicated experiments. Imp. Bur. Soil Sci. Tech. Comm. No. 10.
- HAMILTON, M. A. (1930).—Notes on the culturing of insects for virus work. *Ann. Appl. Biol.* 17: 487-92.
- HILL, A. V. (1941).—Yellow dwarf of tobacco in Australia. II. Transmission by the Jassid *Thamnotettix argentata* (Evans). *J. Coun. Sci. Industr. Res. Aust.* 14:181-6.
- KRISTOFFERSEN, K. B. (1921).—On the relation between sugar content and winter rot in the garden carrots. *Bot. Notiser* 4: 149-63.
- MURPHY, P. A., and M'KAY, R. (1926).—Methods for investigating the virus diseases of the potato, and some results obtained by their use. *Sci. Proc. Roy. Dublin Soc.* 18: 169-84.
- POUND, G. S., and CHAPMAN, R. K. (1947).—Control of aster yellows in carrots by control of *Macrostele divisus* with DDT. (Abstract in *Phytopathology* 37: 18.)
- RIDGWAY, R. (1912).—"Color Standards and Color Nomenclature." (Washington, D.C.)
- SEVERIN, H. H. P. (1930).—Carrot and parsley yellows transmitted by the six-spotted leaf-hopper, *Cicadula sexnotata* (Fall.). *Phytopathology* 20: 920-1.
- SEVERIN, H. H. P. (1932).—Transmission of carrot, parsley and parsnip yellows by *Cicadula divisa*. *Hilgardia* 7: 163-79.
- SEVERIN, H. H. P., and FREITAG, J. H. (1938).—Western celery mosaic. *Ibid.* 11: 495-558.
- SMITH, K. M. (1937).—"A Textbook of Plant Virus Diseases." (Churchill: London.)
- SNEDECOR, G. W. (1940).—"Statistical Methods." 3rd. Ed. (Collegiate Press. Inc.: Ames.)
- STANLEY, W. M. (1936).—Chemical studies on the virus of tobacco mosaic. VI. The isolation from diseased Turkish tobacco plants of a crystalline protein possessing the properties of tobacco mosaic virus. *Phytopathology* 26: 305-20.
- STUBBS, L. L. (1946).—A simple hand duster for the application of abrasives and insecticidal dusts in plant virus transmission studies. *J. Aust. Inst. Agric. Sci.* 12: 53-4.
- STUBBS, L. L., and GRIEVE, B. J. (1944).—A new virus disease of carrots. *J. Vict. Dep. Agric.* 42: 411-2, 415.
- TAYLOR, G. G. (1945).—Preliminary field trials with DDT and 666 against insect pests. *N.Z. J. Sci. Tech.* 27: 129-33.
- THEOBALD, F. V. (1927).—"The Plant Lice or Aphididae of Great Britain." Vol. 2. (Headley: London.)
- THORNBERRY, H. H. (1935).—Effect of phosphate buffers on infectivity of tobacco-mosaic virus. *Phytopathology* 25: 618-27.
- WARINGTON, K. (1940).—The growth and anatomical structure of the carrot (*Daucus carota*) as affected by boron deficiency. *Ann. Appl. Biol.* 27: 176-83.
- WATSON, M. A. (1940).—Studies on the transmission of sugar-beet yellows virus by the aphid *Myzus persicae* (Sulz.). *Proc. Roy. Soc. B* 853: 535-52.
- ZUNDEL, G. L. (1929).—Yellows (virus) on various plants. *Plant Dis. Repr.* 13: 174.

EXPLANATION OF PLATES 1-4

PLATE I

- Fig. 1.—Leaflets from infected carrots showing various stages of mosaic mottling and marginal reddening. The leaflet in the top left-hand corner is from a healthy plant.
- Fig. 2.—Above, leaflets from a healthy carrot plant. Below, leaflets from an infected plant of the same age showing twisting of petioles and subpetioles, and progressive increase in intensity of mosaic mottling with age of leaves (from left to right).
- Fig. 3.—Left, healthy carrot leaf. Right, infected leaf showing "S"-shaped bending of the petiole.
- Fig. 4.—A recently infected mature plant showing rosetting of the youngest leaves.

PLATE 2

- Fig. 1.—Left, dwarfed experimentally infected carrot plant. Right, healthy control plant of the same age.
- Fig. 2.—Infected and healthy slender celery showing, left, petiole epinasty and recurving of younger leaves 21 days after inoculation with infective aphids.

PLATE 3

- Fig. 1.—Transmission of the virus to carrot by core grafting, showing graft union. The plant on the left was grafted with a core from a healthy root and that on the right with a core from an infected root.
- Fig. 2.—*Cavariella aegopodii* feeding on a carrot leaf. Inset, a single aphid. x 10.
- Fig. 3.—Section of a carrot leaf petiole showing the stylet of *Cavariella aegopodii* entering the phloem tissue. x 38.

PLATE 4

- Fig. 1.—Muslin covered plots in which healthy and infected carrot roots were grown for seed production experiments.
- Fig. 2.—Carrot seed plants grown from infected (left) and healthy (right) transplanted roots.
- Fig. 3.—Typical specimens of two virus tolerant carrot varieties of local origin. Left, Burnley selection of a Victorian strain; right, a Western Australian variety, Champion Intermediate.

THE EFFECTS OF PHOSPHORUS SUPPLY ON THE RATES OF INTAKE OF PHOSPHORUS AND NITROGEN AND UPON CERTAIN ASPECTS OF PHOSPHORUS METABOLISM IN GRAMINEOUS PLANTS

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Summary

For experiments previously described, new data are presented relating to the intake of phosphorus by gramineous plants, its distribution within the plant, and its partition in the leaves between alcohol-soluble, nucleic-acid, and "residual" phosphorus.

Major determinants of the rate of intake of phosphorus by the plant are: (*a*) the demand set up by the growth and normal functioning of various plant parts, and (*b*) the concentration of the nutrient in the medium.

It is considered that the indirect effect of phosphorus treatment on growth, and hence on demand, is more important than the direct effects of external concentration of phosphorus on the rates of intake of that nutrient.

Even when growth is not limited by the supply of phosphorus, the rate of intake is limited by the maximal capacity of successive plant parts for accumulation.

For the oat experiment, rates of intake of phosphorus and nitrogen are expressed per unit weight of root system. With nitrogen, the supply of which was the same for all treatments, large initial effects of phosphorus treatment on the rates of intake are accounted for in terms of differences in the ratios of roots to shoots.

Phosphorus-deficient oat plants derived only 30 per cent. of their inflorescence phosphorus from other plant parts; those with an excessive supply derived no less than 93 per cent. of their inflorescence phosphorus from these sources.

The percentage phosphorus contents of the stems, leaves, and roots fell ultimately to lower values with a moderate supply of phosphorus than they did with the deficient supply. A more efficient re-utilization of phosphorus in the plants receiving the greater supply is favoured as an interpretation of this "dilution" effect.

From an examination of the data for protein-nitrogen and nucleic-acid phosphorus in the leaves of young plants, it is concluded that the effects of phosphorus treatment on protein-nitrogen content at this stage are due primarily to variation in nucleoprotein content.

With phosphorus deficiency, oat seedlings soon exhausted their seed reserves of phosphorus, and this was followed by a drastic change in the partitioning of leaf phosphorus, such that absolute nucleic-acid phosphorus was reduced to one-fifth of the value obtaining eleven days earlier, alcohol-soluble phosphorus decreased slightly, and the water-soluble fractions represented by "residual" phosphorus increased by 30 per cent. Total phosphorus was virtually unchanged in amount. During this same period of eleven days, the whole of the phosphorus intake from the medium was retained by the roots, and there was a stimulation of root growth relative to leaf growth. These facts suggest that the high root weight ratios found with phosphorus deficiency may be due to the fixation in organic forms of a greater proportion of the absorbed phosphorus, so that relatively little is available for shoot growth.

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Nucleo-cytoplasmic ratios of oat and *Phalaris* leaves are presented for a wide range of growth stage and nutrient treatment. No obvious correlations with growth data such as the relative leaf growth rates were revealed.

The literature relating to the partitioning of phosphorus within plant tissues is discussed and, where comparisons are relevant, this is in substantial agreement with the work reported here.

The theory of biological explanation is considered in relation to certain growth phenomena, and it is indicated that explanation should be sought at the biological as well as at the physico-chemical level of organization.

I. INTRODUCTION

The data presented in this paper all derive from two experiments in which phosphorus supply has been varied, and plants have been harvested and examined at a number of growth stages. The data for the main experiment — that with oats — have already been examined with respect to quantitative growth analysis (Williams 1936, 1939), the intake and distribution of nitrogen within the plant (Williams 1938), and the respiration of the leaves (Petrie and Williams 1938): the new data relate to the intake of phosphorus itself, its distribution within the plant, and its partition between inorganic and certain groups of organic compounds. As no extension of this work is contemplated, it is desirable to attempt some synthesis of the work, and to extract some general principles.

Additional data are also presented for an experiment with *Phalaris tuberosa* L., when two levels of phosphorus and nitrogen were supplied in all combinations. The growth analysis of this experiment appears elsewhere (Williams 1946).

In a general discussion of the upward movement and distribution of solutes, Hoagland (1944) stresses the need to envisage the plant as an integrated organism. Thus the growth and functioning of the roots are dependent upon metabolites normally deriving from the shoot. Likewise the distribution of solutes is not merely a necessary consequence of the movement of water containing these solutes. This latter point was illustrated by reference to work of Arnon, Stout, and Sipes (1940), where radioactive phosphate was used in a study of the movement of ions to developing fruit of the tomato plant. Treatment was applied when the plants already bore fruits of all sizes, and it was found that the radioactive phosphate accumulated to the greatest extent in the young growing fruit, and not in the regions of highest water loss. Here the control seemed to reside in the meristematic tissues having a high rate of metabolism.

Petrie (1934) also discusses the accumulation and redistribution of nutrients within the plant, and he contrasts the behaviour of potassium and calcium in these respects. The later-formed organs derive part, if not all, of their potassium from those formed earlier, but supplies of calcium are usually derived only from the external medium. The difference is attributed to the lesser mobility of the calcium ion, and to its almost irreversible chemical fixation in the tissues. Loss of potassium from a given organ and from the plant is thought to commence with the attainment of the maximum sap concentration attainable under the conditions, and this maximum is believed to decline with age. Petrie, however, is not concerned with the case where potassium is in short supply; under these

conditions he recognizes that the transfer of ions to other cells and organs might prevent the attainment of this maximum sap concentration.

The behaviour of nitrogen and phosphorus with respect to their accumulation and redistribution is undoubtedly more complex than that of potassium or calcium. The former are fixed organically in a variety of compounds, and this fixation is quite definitely reversible. An important point of difference between phosphorus and nitrogen is that, relatively speaking, the cell is able to accumulate much larger amounts of inorganic and soluble-organic compounds of phosphorus. To this extent phosphorus may have some features in common with potassium. Be that as it may, there would seem to be considerable scope for integrated control of the movement of nitrogen and phosphorus within the plant, and an attempt is made to develop this concept of integrated control in relation to the data presented here. The concept itself is consistent with Frank's (1935) definition of the problem of organic growth as "the measurement of the changing dimensions of structure-function activity of the organism, and the discovery of the sequence in which these changes occur."

The later sections of this paper are largely devoted to the discussion of alcohol-soluble, nucleic-acid, and residual phosphorus data for the leaf material of the two experiments mentioned above. This part of the paper could perhaps have been treated as an independent unit, though the data are relevant to an understanding of the redistribution of phosphorus within the plant. A review of the literature relating to the fractionation or "partition" of phosphorus within plant tissues is presented as part of the general discussion.

Details of the two experiments have been given before (Williams 1936, 1946); only the bare essentials are given here. The experimental plants were grown, five per pot, in water-washed sand (14 and 15 kg. per pot in the respective experiments) and with abundant water supply. In the oat experiment, phosphorus was applied as sodium acid phosphate. Nitrogen and phosphorus were applied to *Phalaris* as calcium nitrate and calcium acid phosphate respectively, calcium being then equalized for all treatments by the addition of appropriate amounts of calcium chloride.

The amounts per pot were:

A. Algerian Oats (*Avena sativa* L.)

Treatment I (P_1) 0.008 g. P per pot.

" II (P_2) 0.09 " " " "

" III (P_3) 0.6 " " " "

B. *Phalaris tuberosa* L.

Treatment	P	N	Cl
P_1N_1	0.04 g.	0.4 g.	3.15 g. per pot.
P_1N_2	0.04 "	1.6 "	2.05 " " "
P_2N_1	0.24 "	0.4 "	3.06 " " "
P_2N_2	0.24 "	1.6 "	1.97 " " "

The treatment terminology, P_1 , P_2 , etc., has been adopted as being more descriptive than that previously used.

Since the greater part of the discussion centres around the oat experiment, it will be helpful to give a statement of the stages of development reached by the plants at the successive harvests.

Harvest 1.—First leaf well through the coleoptile and developing rapidly.

Harvest 2.—First leaf mature (i.e. attained maximum area) and second leaf just showing.

Harvest 3.—First and second leaves mature and third leaves showing in many cases. Tillering commencing, especially in P_2 and P_3 .

Harvest 4.—Fourth leaf mature and fifth leaf developing. Several tillers per plant, with more in P_2 and P_3 than in P_1 .

Harvest 5.—Leaves at peak of their development and stems growing rapidly.

Harvest 6.—Flowering in P_1 but post-flowering in P_2 and P_3 .

Harvest 7.—Full maturity, leaves dead.

II. ANALYTICAL METHODS

Total phosphorus was estimated by the colorimetric method of Zinzadze (1931, 1935) after wet-ashing with nitric acid and perhydrol. In more recent work perchloric acid has been used in place of perhydrol. Appreciable blue colouration was found in blank estimations, and this was traced to siliceous impurities in the sodium bicarbonate (or anhydrous carbonate) used to neutralize the solutions prior to treatment with sodium metabisulphite. A constant excess of bicarbonate was therefore added, and the solutions were back titrated with normal sulphuric acid.

Methods for the estimation of the phosphorus-containing organic compounds of biological materials are far from satisfactory, and this in spite of their physiological importance. However, the method of Javillier and Allaire (1931) and Javillier and Colin (1933) for the estimation of nucleic-acid phosphorus (thought to be desoxyribose plus ribose nucleic acid) has been modified (Williams 1945) and was used in the present work.

Since this method requires the pre-extraction of the plant material with alcohol, the alcohol-soluble phosphorus was also determined. As a measure of phosphatide phosphorus, this latter is admittedly crude, for it has been shown by Jordan and Chibnall (1933) that considerable quantities of non-phosphatide phosphorus may be extracted by this solvent. More recent work by Rewald (1936, 1937*a*, 1937*b*) points to the inadequacy of any one solvent or solvent mixture for the extraction of phosphatides, and his work on "free" and "bound" phosphatides offers new opportunities in the study of phosphorus metabolism.

In the present investigation, the oven-dry leaf samples were extracted for 15 hours with absolute alcohol in Soxhlet extractors. The capacity of the upper vessels of the latter was 65 ml., and the siphoning rate approximately 9 times per hour. The selection of a 15-hour extraction period was arbitrary, for it was found that additional small increments of phosphorus were extractable up to, and probably beyond, a total period of 30 hours. In the samples tested, however, the amount of phosphorus extracted during the first 7.5-hour period was of the

order of ten times the amount extracted during the second period. The phosphorus contents of all extracts were determined by the method of Zinzadze (loc. cit.).

Jordan and Chibnall (1933) used ether alone for extracting phosphatides; thus a comparison of the alcohol- and ether-soluble phosphorus of 6 samples of leaf material from the present oat experiment is of interest (see Table 1). The extraction period was the same for each solvent. In rapidly-growing leaf material (harvest 4) the relative amount extractable with ether was negligible, whereas it increased to about 25 per cent. in senescent leaves (harvest 6). If these dis-

TABLE 1
ALCOHOL- AND ETHER-SOLUBLE PHOSPHORUS IN OAT LEAVES*

Solvent	Harvest 4		Harvest 5		Harvest 6	
	P ₂	P ₃	P ₂	P ₃	P ₂	P ₃
Anhydrous ether, E	4**	7**	8.2	10.7	8.1	25
Absolute alcohol, A	260	410	86	135	33	94
E/A x 100	<2	<2	9.5	7.9	24.5	26.6

* Parts per million. ** Very approximate values.

crepancies in time and between solvents are attributable mainly to "the extraction of water-soluble phosphorus by the boiling alcohol," as suggested by Jordan and Chibnall, then the data presented here are worthless as indices of phosphatide content. It is possible, however, that the bulk of the phosphatides present were insoluble in anhydrous ether, and this interpretation gains support from the finding of Halpern (1945) that phosphatides are so strongly held by proteins that certain solvents including ether do not extract any phosphatide at all from salmon roe. In either case the data must be interpreted with caution.

A third and very arbitrary phosphorus fraction, here styled "residual" phosphorus is obtained by the difference between total phosphorus and the sum of alcohol-soluble and nucleic-acid phosphorus. It is an index of those compounds which occur primarily in the aqueous phases of the cell, and would include inorganic and hexose-phosphates.

The method used for the estimation of protein nitrogen is described by Petrie (1937), the precipitant being tannic acid.

III. PRESENTATION OF DATA AND DISCUSSION

The data relating to the intake and distribution of phosphorus within the plant as affected by phosphorus supply and stage of growth are summarized in the lower portion of Figure 1, where the absolute phosphorus contents of the roots, stems, leaves, and inflorescences of oats are plotted additively. The primary data are presented in Table 4, and parallel data relative to nitrogen for this same experiment are presented elsewhere (Williams 1938, Fig. 5 and Tables 3, 4, and 7).

(a) The Rate of Intake of Phosphorus

Watson and Petrie (1940, p. 331), when discussing the intake of nitrogen by the tobacco plant, visualized three major determinants of the rates: (a) the external concentration or supply of the nutrient; (b) the capacity of the roots

for intake; and (c) the capacity of the tissues as sinks for the nutrient. The third of these determinants may be restated as the demand for the nutrient set up by the growth and normal functioning of various plant parts, including the roots.

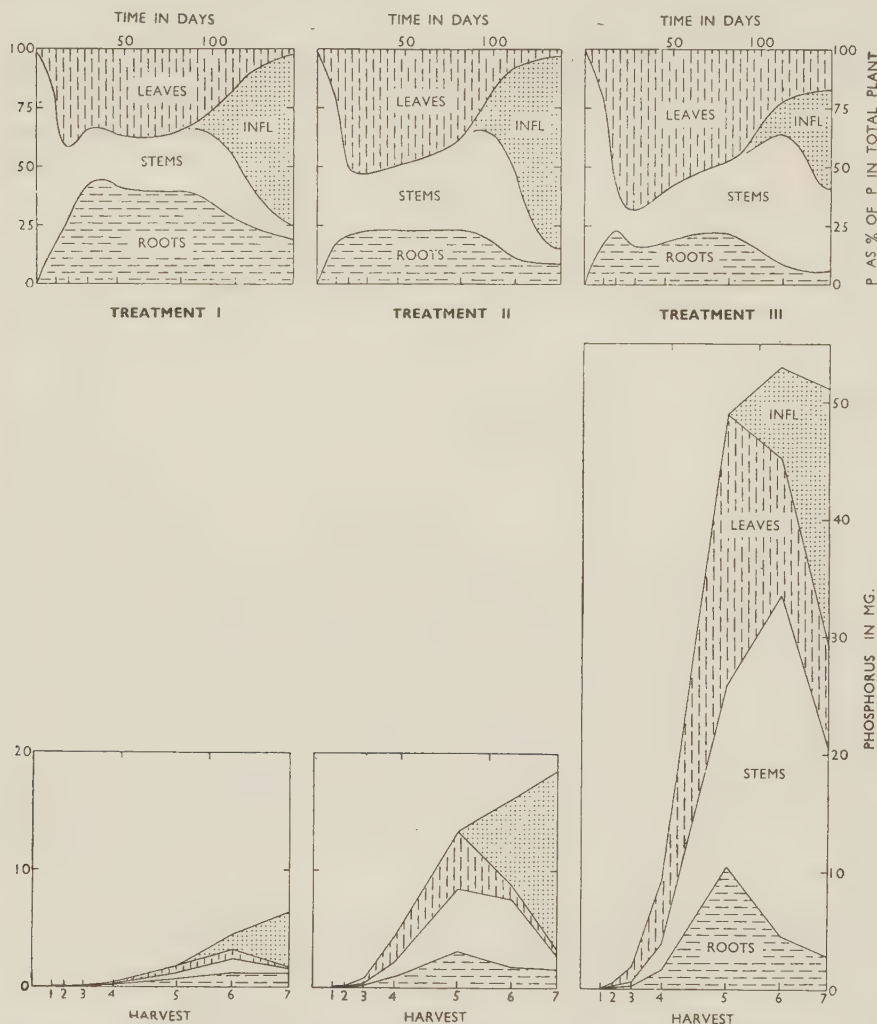


Fig. 1.—The intake of phosphorus by oats and its distribution within the plant. Above: The phosphorus present in different parts expressed as percentage of the total phosphorus in the plant. Below: The absolute phosphorus content of the whole plant and its parts. The treatments are low (P_1), medium (P_2), and high (P_3) supplies of phosphorus respectively.

In this form it also covers the second determinant, at least in the sense that this is developed by Watson and Petrie. It is considered that the influence of the external concentration of the nutrient on intake is more often subject to the controlling influence of the growth factors than otherwise; for this reason the internal or growth factor is considered first. The mean rates of intake of phosphorus and nitrogen per day are presented in Table 2.

(i) *The Internal or Growth Factor*

Each vegetative organ of a plant passes through phases of intake, relatively constant content, and of export of many of its mineral constituents.* Each organ thus has a certain capacity to accumulate nutrients such as phosphorus, and in its senescent phase it constitutes a potential source of these nutrients for younger plant parts. That the demand of younger organs for nitrogen must be regarded as the main factor causing loss of nitrogen from mature leaves has been suggested for barley by Walkley (1940), and for tobacco by Watson and Petrie (1940), and it is probable that export of phosphorus is initiated in the same way. At the same time, the demand of each organ for phosphorus is usually met in part, if

TABLE 2
MEAN RATES OF INTAKE* OF PHOSPHORUS AND NITROGEN BY OATS AS AFFECTED BY
PHOSPHORUS SUPPLY AND AGE

Harvest Interval	Phosphorus			Nitrogen		
	P ₁	P ₂	P ₃	P ₁	P ₂	P ₃
1-2	-0.0004	0.0182	0.059	0.122	0.154	0.149
2-3	0.0023	0.058	0.131	0.170	0.416	0.325
3-4	0.019	0.208	0.441	0.474	2.80	2.65
4-5	0.039	0.250	1.098	1.58	2.08	2.21
5-6	0.087	0.087	0.129	0.93	-0.09	0.01
6-7	0.057	0.095	-0.067	-0.02	0.02	-0.20

* Mg. per day.

not entirely, by absorption from the external medium; this rate of intake by the roots, however, is restricted to the extent that phosphorus is more readily available within the plant itself.

Evidence for this general scheme may be adduced from the present experiment. Thus, during the early development of the seedling, the grain is the sole source of phosphorus, and for some time it remains an important alternative source, especially if the external medium is deficient in phosphorus. Between harvests 1 and 2 (days 11-18), the leaves and roots of P₁ derived all their phosphorus from the "stems" (which included the grain); whereas with P₂ and P₃, the "stem" fractions themselves showed net gains of phosphorus.

During the phase of rapid vegetative growth (between harvests 3 and 5), the roots, stems, and leaves all accumulated phosphorus rapidly, and the influence of external supply is very evident (see the absolute increments of Table 3). As between P₁ and P₂, however, much of the difference in intake is attributable to the very much greater rate of growth of the plant parts with P₂. This will be evident from an inspection of the increments of phosphorus per unit increase in dry weight of the parts (Table 3). Thus for the leaves of harvest-interval 3-4,

* In this discussion it is understood that we are concerned only with net change over periods of several days, though the same principles would doubtless hold for the interpretation of diurnal fluctuations such as those suggested by the work of Phillis and Mason (1942a) with leaves of the cotton plant. By contrast, studies with radioactive phosphorus (e.g. Biddulph 1941) are concerned with rates of translocation as such.

the absolute increase was more than fourteen times greater with P_2 than it was with P_1 , but the increase per unit increase of dry matter was little more than twice as great with P_2 as it was with P_1 . For interval 4-5 this treatment difference would seem to be due entirely to the growth factor, all three rates of increase per unit increase in dry weight being lower with P_2 than with P_1 . With P_3 , the rates of growth of the parts were little if at all greater than those with P_2 (Williams 1936), so that practically all of the difference in intake of phosphorus over this period could perhaps be attributed to differences of external supply. Even so, it is noteworthy that the absolute phosphorus increments for the three plant parts of P_3 are almost proportional to their respective weight increments, the increments per gram increase in dry weight being approximately equal within each harvest-interval, whereas they vary greatly in P_2 . This suggests that a general state of

TABLE 3
INCREMENTS IN ABSOLUTE PHOSPHORUS CONTENT OF THE LEAVES, STEMS, AND ROOTS
OF OATS FOR HARVEST-INTERVALS 3-4 AND 4-5

Increments (mg.)	Interval 3-4 Days 29-46			Interval 4-5 Days 46-82		
	P_1	P_2	P_3	P_1	P_2	P_3
Leaves	0.119	1.72	4.29	0.471	2.83	17.55
Stems	0.070	1.00	1.82	0.381	4.11	13.13
Roots	0.131	0.82	1.39	0.546	2.05	8.84
Increments (mg. per g. increment in dry weight)						
Leaves	1.37	2.99	7.58	0.61	0.50	2.74
Stems	2.45	4.38	7.26	1.00	0.88	2.79
Roots	1.31	3.56	7.10	0.93	0.69	2.31

saturation with phosphorus exists in the tissues, and that the sap concentration may be approaching the maximum attainable under the conditions (Petrie 1934, see above). In these circumstances phosphorus should be present in sap-soluble form to a relatively greater extent in P_3 than in P_2 , and this is supported by the data for "residual" phosphorus in the leaves (see Table 6), "residual" phosphorus being an index of the phosphorus compounds of the aqueous phases of the cell. At all harvests, "residual" phosphorus as a percentage of total phosphorus is greater with P_3 than with P_2 , and for harvests 3, 4, and 5 the mean values are 85 and 94 per cent. for P_2 and P_3 respectively. Under such conditions of saturation, the rate of intake of phosphorus with P_3 would be determined by the maximal capacities of the plant parts to accumulate this element, rather than by any direct effect of its concentration in the medium.

It will be noted that for P_1 and P_2 at both harvest-intervals the increments of phosphorus per unit increase in dry weight are greatest in the stems. This perhaps indicates a relatively greater meristematic activity in stem tissues over this period, when shoot elongation and inflorescence differentiation are proceeding. That a similar effect is not in evidence for P_3 is in accord with the suggestion of the last paragraph, that the rates of intake in this case are primarily determined by the maximal capacities of the several parts to accumulate phosphate in the cell sap.

The cessation of nitrogen intake (at harvest 5 with P_2 and P_3 and at harvest 6 with P_1) is not synonymous with the loss of capacity for intake in general, for phosphorus intake persisted to full maturity (harvest 7) with P_1 and P_2 . Crowther (1934) found that nitrogen intake ceased at the time of rapid development of the inflorescence, and he suggested that competition for supplies of carbohydrate stopped the growth of the roots, and with it the intake of nitrogen.

In the present experiment, nitrogen intake did cease concurrently with the rapid development of the inflorescence and with the cessation of root growth as measured by dry weight change. However, the exhaustion of the supply of nitrogen was almost certainly the cause of the cessation of nitrogen intake in P_2 and P_3 , and for P_1 an internal mechanism unrelated to carbohydrate supply has already been suggested (Williams 1938, pp. 78-9). This mechanism will be discussed, as it applies to phosphorus re-utilization, in a later section of this paper.

For phosphorus, and to a lesser extent for nitrogen, it would seem that the growth factor was the primary determinant of continued intake after the cessation of root growth.

(ii) *The External Factor of Supply*

The external concentration of phosphorus in sand cultures may not be related in any simple manner to the amounts of phosphorus added in solution, for it has been shown by Dunlap (1939) that phosphate retention is an important phenomenon in certain sands, and that the amount of this retention is determined by the kind of sand, the concentration of added phosphate, and the duration of treatment. Furthermore, it is now known that the total phosphorus content of the washed river-sand used for the present experiment with oats may have been of the same order as that applied as the highest treatment (600 mg. P per pot). Fortunately, little of this was available to the plants, since only 19.2 mg. of phosphorus per pot were absorbed by plants of P_1 . It should be noted, however, that only 8 mg. were supplied in the culture solution and 0.5 mg. in the seeds, so that almost 11 mg. of phosphorus were derived from the sand. The ratios of maximum phosphorus absorbed to phosphorus supplied were 2.4, 0.61, and 0.27 for P_1 , P_2 , and P_3 respectively.

With P_1 , the rate of phosphorus intake per day rose slowly to a maximum at harvest-interval 5-6, with the result that 71 per cent. of its total intake occurred after harvest 5, and 29 per cent. after harvest 6. With P_2 and P_3 , only 27 and 8 per cent. respectively of the intake occurred after harvest 5, and rates of intake per day were at a maximum for harvest-interval 4-5. It is clear from the data that the presumed external concentrations of phosphorus were important, even if indirect, determinants of the rates of intake per day for the first half of the growing period.

(iii) *The Rate of Intake of Phosphorus per Unit Weight of Root*

Rates of nutrient intake per day are complicated by the size factor of the absorbing system; rates per unit weight of root are therefore presented as crude indices of the rates per unit area of absorbing surface. Kreyzi (1932) used the root weight basis in his study of the intake of phosphorus by plants in water cultures.

The instantaneous rate of intake of a mineral nutrient M is given by the equation,

$$I_M = \frac{1}{R} \frac{dM}{dt},$$

where R is the dry weight of the root system at that instant. The mean value of I_M for a finite time-interval may be calculated from the approximate formula

$$I_M = \frac{\log_e R_2 - \log_e R_1}{t_2 - t_1} \times \frac{M_2 - M_1}{R_2 - R_1}.$$

The errors involved in the use of this formula are discussed by Williams (1945) in connection with the parallel equation for net assimilation rate.

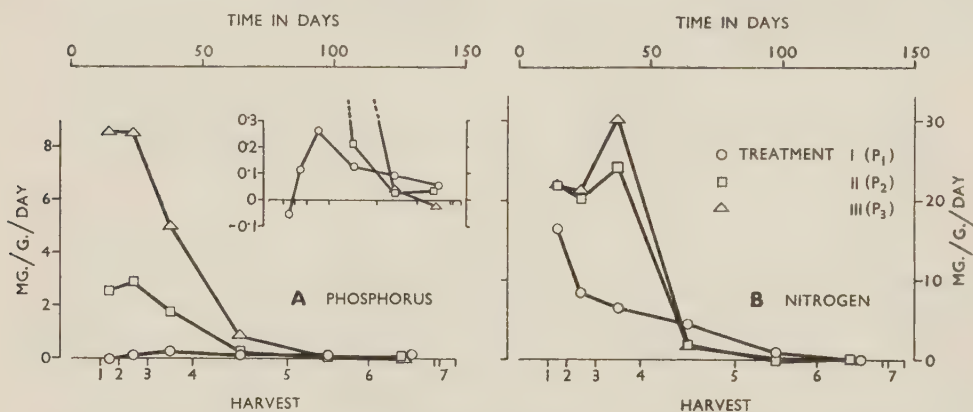


Fig. 2.—Rates of intake of phosphorus (A) and nitrogen (B) per unit weight of root in oats.

The values of I_M for phosphorus are presented in Figure 2A and the time trend for P_1 is shown more clearly in the inset of that figure. With this treatment, the rate rose from an initial negative value to a maximum of only 0.26 mg. per g. per day at harvest-interval 3-4. Thereafter the rate of intake fell steadily, and after harvest 5, became greater than the rates for P_2 and P_3 . While the negative value requires experimental confirmation, it is conceivable that the internal concentration of phosphate in the roots (being derived from seed reserves) was so great that some was lost to the phosphorus-deficient medium. The rapid fall in the phosphorus content of all plant parts (see Table 4) and the presumed fall in root phosphate concentration would account for the observed upward trend in the rate of intake from the deficient medium. With P_2 there was a slight increase in the rate to a maximum of 2.82 mg. per g. per day at interval 2-3, but there was no increase in the rate with P_3 . The importance of the concentration of phosphorus in the medium as a determinant of the rate of intake is again evident, even though the integrated control of the rate by the organism itself has been shown to be of primary significance.

The relatively high rates of phosphorus intake with P_1 for intervals 5-6 and 6-7 are an expression of the demand for inflorescence phosphorus (see later), though the high nitrogen status of plants of P_1 may also have played a part in maintaining these rates.

(b) The Rate of Intake of Nitrogen per Unit Weight of Root

This section properly belongs to an earlier paper (Williams 1938), but is included here because the basis of expression was not then considered. It is of special interest because it provides a definite example of control by the growth factor.

TABLE 4

ABSOLUTE AND RELATIVE AMOUNTS OF TOTAL PHOSPHORUS IN THE LEAVES, STEMS, ROOTS, AND INFLORESCENCES OF OATS

Harvest		Phosphorus (% Dry Weight)			Absolute Phosphorus (mg.)		
		P ₁	P ₂	P ₃	P ₁	P ₂	P ₃
Leaves	1	0.596	0.596	0.596	0.0215	0.0215	0.0215
	2	0.296	0.784	1.87	0.0403	0.115	0.295
	3	0.140	0.743	2.27	0.0419	0.455	1.323
	4	0.138	0.342	0.899	0.161	2.17	5.61
	5	0.071	0.080	0.330	0.632	5.00	23.16
	6	0.051	0.029	0.240	0.765	1.24	11.69
	7	0.016	0.014	0.199	0.170	0.56	8.82
Stems	1	0.532	0.532	0.532	0.0606	0.0606	0.0606
	2	0.405	0.777	1.11	0.0304	0.653	0.097
	3	0.255	0.882	1.38	0.0288	0.212	0.313
	4	0.247	0.482	0.779	0.099	1.21	2.13
	5	0.114	0.108	0.307	0.480	5.32	15.26
	6	0.042	0.045	0.221	1.184	5.76	28.95
	7	0.014	0.012	0.166	0.407	1.29	17.70
Roots	1	0.365	0.365	0.365	0.0172	0.0172	0.0172
	2	0.232	0.459	1.23	0.0258	0.0459	0.117
	3	0.153	0.529	1.34	0.513	0.193	0.310
	4	0.137	0.380	0.776	0.182	1.01	1.70
	5	0.101	0.095	0.261	0.728	3.06	10.54
	6	0.103	0.061	0.158	1.246	1.78	4.55
	7	0.124	0.066	0.121	1.288	1.57	2.81
Inflorescences	6	0.152	0.178	0.233	1.338	7.28	7.76
	7	0.170	0.193	0.294	4.590	15.02	21.81
Whole Plant	1				0.0993	0.0993	0.0993
	2				0.0965	0.2264	0.509
	3				0.122	0.860	1.946
	4				0.442	4.39	9.44
	5				1.84	13.38	48.96
	6				4.533	16.06	52.95
	7				6.40	18.44	51.14

In spite of the fact that the supply of nitrogen was initially the same for each treatment, the data of Figure 2B show that phosphorus deficiency greatly depressed the rate of intake of nitrogen. The depression was most pronounced for harvest-interval 3-4. The subsequent reversal of this effect was due to the exhaustion of their nitrogen supply by the larger plants of P₂ and P₃. There was also an increase in the rate of intake with P₃ over that with P₂ for harvest-interval 3-4.

The early effects of treatment on nitrogen intake, however, were not primarily due to any direct effects of phosphorus on the mechanism of ion intake, but to its differential effect on the growth of roots and shoots. For the plants as a whole, the rates of increase of nitrogen per unit increase in dry matter were similar for all treatments (an average of 40.7, 47.1, and 45.1 mg. nitrogen per g. dry matter formed for P_1 , P_2 , and P_3 respectively over harvest-interval 1-4), but the implied uniform demands for nitrogen were met via the root system which varied greatly in size (dry weight) relative to the size of the whole plant. Thus, assuming a uniform requirement of 46.2 mg. (the value for P_2) per g. dry matter added over harvest-interval 3-4, the rates of intake per g. of root system would be 8.1, 24.3, and 31.6 mg. nitrogen per day for P_1 , P_2 , and P_3 respectively. The actual rates were 6.6, 24.3, and 30.4 mg. per g. per day. The further slight depression below theoretical with P_1 could be associated with depressed protein synthesis and the piling up of soluble nitrogen compounds; this occurred in the leaves (Williams 1938) and may be inferred for the roots also.

(c) *The Redistribution of Phosphorus within the Plant*

Reference to Figure 1 and Table 4 shows that, with all treatments, phosphorus accumulates rapidly in the roots and leaves, then in the stems, and finally in the inflorescences, and this is accomplished with varying degrees of redistribution of phosphorus. Reference has been made to the grain as a source of phosphorus for the seedling, and to the fact that each successive vegetative organ becomes a potential source of phosphorus for younger plant parts. In this way phosphorus is redistributed within the plant, and may be re-utilized many times during the life of an annual plant. Reid (1941) has shown that cells only 15 mm. from the root tips of cowpea seedlings may be losing phosphorus in this manner. Later the inflorescence, with its very great demand for phosphorus, becomes of increasing importance as a determinant of the redistribution of the element within the plant. The magnitude of this demand in oats may be gauged from the fact that the amounts of phosphorus in the inflorescence, expressed as percentages of the amounts in the whole plant at maturity, were 72, 82, and 43 for P_1 , P_2 , and P_3 respectively.

In Table 5, the net increments of phosphorus in leaves, stems, roots, and inflorescences are presented for harvest-intervals 5-6 and 6-7. For P_1 , interval 5-6, all plant parts show net increases, and the inflorescences account for almost 50 per cent. of the total intake of phosphorus. In the sense that there was no net loss of phosphorus from the other plant parts, it may be postulated that the inflorescence phosphorus was derived entirely from the external medium. The picture is more complex with P_2 and P_3 over this interval; leaves and roots showed net losses of phosphorus, and stems and inflorescences net gains. In P_2 the stems received a total of 7.72 mg. of phosphorus: 5.04 mg. from the leaves and roots, and 2.68 mg. from the medium. Thus the proportion of stem and inflorescence phosphorus derived from the external medium may be regarded as 2.68/7.72 or 35 per cent. Similarly for P_3 the proportion becomes 3.99/21.45 or 19 per cent.

For harvest-interval 6-7, the leaves, stems, and roots of all treatments were losing phosphorus, hence there was no competition for such phosphorus as was

derived from the medium. For P_1 and P_2 , the relative amounts of inflorescence phosphorus derived from the medium were 57 and 31 per cent. respectively. For P_3 , however, all of the inflorescence phosphorus came from other plant parts, and the data indicate that some phosphorus may have been lost to the medium.

TABLE 5

INCREMENTS IN ABSOLUTE PHOSPHORUS CONTENT OF THE LEAVES, STEMS, ROOTS, AND INFLORESCENCES OF OATS FOR HARVEST-INTERVALS 5-6 AND 6-7

	Interval 5-6 (days 82-113)			Interval 6-7 (113-146) (113-138) (113-140)		
	P_1 (mg.)	P_2 (mg.)	P_3 (mg.)	P_1 (mg.)	P_2 (mg.)	P_3 (mg.)
Leaves	0.13	-3.76	-11.47	-0.60	-0.68	-2.87
Stems	0.70	0.44	13.69	-0.77	-4.47	-11.25
Roots	0.52	-1.28	-5.99	-0.02	-0.21	-1.74
Inflorescences	1.34	7.28	7.76	3.25	7.74	14.05
	(100)	(35)	(19)	(57)	(31)	(0)
Whole plant	2.69	2.68	3.99	1.86	2.38	-1.81

The italic figures are estimates of the percentage amounts of inflorescence phosphorus derived from the medium (for explanation see text).

While the above analysis makes certain assumptions by virtue of the fact that only net increments are known, it nevertheless provides satisfactory comparative data upon which to build a tentative interpretation relative to the intake and distribution of phosphorus. Thus, in all, phosphorus-deficient plants derived only 30 per cent. of their inflorescence phosphorus from other plant parts, whereas those plants which had an excessive supply derived no less than 93 per cent. of their inflorescence phosphorus from these sources. In the latter case it is probable that the supply of phosphorus was still great, but that an abundant and more accessible supply was made available by the senescent breakdown of the protoplasm in the leaves and roots, and later in the stems of these plants. Evidence presented elsewhere (Williams 1938) suggests that this breakdown was caused by the demand of the inflorescence for nitrogen rather than phosphorus; this would contribute to the ready availability of the internal supply of phosphorus, and to conditions favouring a loss of phosphorus to the medium.

With phosphorous deficiency, on the other hand, the inflorescence phosphorus was more readily derived from the deficient medium than from the other plant parts. The nitrogen status was much higher in this case, and such senescent breakdown of the protoplasm as did occur in the leaves etc. may have been initiated by a demand for phosphorus rather than for nitrogen.

(d) *Relative Phosphorus Contents*

These contents (elementary phosphorus per cent. dry weight) are presented in Table 4 and in Figure 3 (leaves only). In leaves, stems, and roots of P_3 these relative contents attained very high maxima (2.27 per cent. for leaves) at harvest 3. For P_2 the maxima were much lower, and for P_1 the values fell rapidly from the

initial value. By harvest 5, however, the values for P_2 had fallen to the same level as those for P_1 , and subsequently they fell still lower, especially in the roots. These phenomena are expressions of the "dilution" effect frequently encountered when a growth response is induced by an additional supply of the nutrient concerned. In these cases, the treatment appears so to stimulate the production of dry matter (primarily carbohydrates and proteins) that the increased quantity of the nutrient taken into the plant is thereby "diluted." Thus, for harvest-interval 4-5 (see Table 3) the intake of phosphorus by the leaves, stems, and roots was from 4 to 10 times greater with P_2 than with P_1 , yet the rates of intake per gram increment of dry matter formed were consistently less with P_2 than with P_1 .

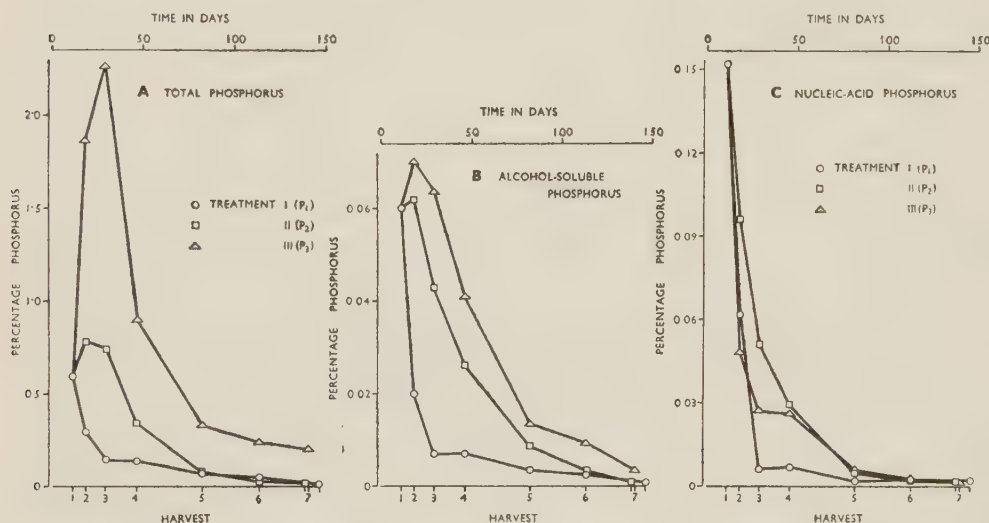


Fig. 3.—Total, alcohol-soluble, and nucleic-acid phosphorus as percentage of dry weight in leaves of oats.

The simplest interpretation of the "dilution" effect is the very literal one, stated above, that the production of carbohydrates etc. is so stimulated by treatment that the percentage content of the nutrient falls more rapidly than in the absence of treatment. Gregory and Richards (1929) found a slight though consistent increase in carbon assimilation with increased phosphorus supply at both low and high light intensities. The barley leaves studied, however, had only just attained maturity (full expansion) and it may be questioned whether they were very deficient in phosphorus. It is probable that carbon assimilation of individual leaves falls off rapidly with time in the case of phosphorus deficiency, as is indicated by measurements of the assimilation of the leaves as a whole. For the present experiment (Williams 1936) it was found that net assimilation rates (dry weight basis) were depressed by phosphorus deficiency during early stages of growth, and that a smaller proportion of the plant was concerned with the production of carbohydrates.

An alternative interpretation would be that the relative shortage of nitrogen with P_2 resulted in an earlier release of phosphorus from senescent plant parts,

and a more efficient re-utilization of this phosphorus. In this case, the demand per unit of new growth for phosphorus from the medium could have been less than with P_1 over the same period, and the phosphorus contents of the plant parts "diluted" by the phosphorus-poor tissues of the senescent individual parts. This second interpretation gains support from the fact that the inflorescences of P_2 were still able to obtain their phosphorus at a greater rate per gram increment of dry matter than was the case with P_1 . These rates for harvest-interval 5-6 were 1.52, 1.78, and 2.33 mg. per g. for P_1 , P_2 , and P_3 respectively; for interval 6-7 the rates were 1.79, 2.10, and 3.43 mg. per g. of dry matter added to the inflorescences.

(e) *Alcohol-Soluble, Nucleic-Acid, and Residual Phosphorus in the Leaves*

The total, alcohol-soluble, and nucleic-acid phosphorus contents (relative) of the leaves are shown in Figure 3, and the absolute data (including residual phosphorus) are presented in Table 6. For reasons discussed (see Section II) a close examination of the time trends and treatment effects on percentage alcohol-soluble phosphorus—a crude index of phosphatide phosphorus—will not be attempted. It is sufficient to point to the general resemblances between these and the data for total phosphorus in the leaves.

TABLE 6

ABSOLUTE AMOUNTS OF NUCLEIC-ACID, ALCOHOL-SOLUBLE, AND RESIDUAL PHOSPHORUS IN THE LEAVES OF OATS ($\mu\text{G.}$)

Harvest	Time in Days	Treatment I P_1			Treatment II P_2			Treatment III P_3		
		Nucleic-Acid P	Alcohol-Soluble P	Residual P	Nucleic-Acid P	Alcohol-Soluble P	Residual P	Nucleic-Acid P	Alcohol-Soluble P	Residual P
1	11	5.5	2.2	13.8	5.5	2.2	13.8	5.5	2.2	13.8
2	18	8.5	2.7	29.1	14.2	9.2	91.8	7.6	11.1	276.0
3	29	1.7	2.1	38.1	31.0	26.6	397.0	15.9	37.1	1270.0
4	46	8.5	8.3	145.0	186.0	167.0	1820.0	165.0	255.0	5190.0
5	82	13.2	30.4	589.0	272.0	541.0	4190.0	382.0	948.0	21800.0
6	113	33.9	37.5	694.0	78.0	142.0	1020.0	120.0	458.0	11100.0
7	138	—	—	—	46.0	32.0	492.0	—	—	—
	140	—	—	—	—	—	—	59.0	155.0	8600.0
	146	21.4	5.3	146.0	—	—	—	—	—	—

The nucleic-acid phosphorus contents, on the other hand, present a strikingly different picture. These values fall rapidly with time with all treatments, and early values for P_3 are markedly depressed below those for P_2 . Between harvests 1 and 3 the value for P_1 falls to a very low figure, but is maintained relatively constant thereafter.

For harvests 2, 3, and 4, striking similarities were noted in the effects of treatment, though not of time, on nucleic-acid phosphorus and protein-nitrogen contents (for protein-N see Williams 1938, Fig. 2, Exp. 2). This relation is shown in Figure 4, where protein-nitrogen and nucleic-acid phosphorus are plotted

respectively as the dependent and independent variables. Linear regressions are shown for each set of three values within harvest.

Two factors favour the suggestion that these approximately linear relations are due primarily to the biochemical association of the variables as nucleoproteins. Firstly, the treatments change position relative to each other from harvest to harvest (Fig. 4): if it were otherwise the relation could be merely an expression of an internal or time factor common to the variables. Secondly, the ratios of

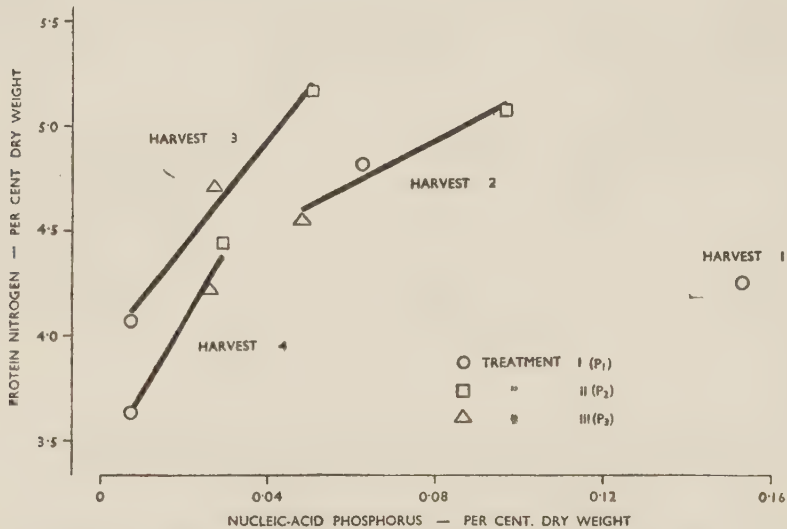


Fig. 4.—The relation of protein nitrogen to nucleic-acid phosphorus in the leaves of young oat plants.

nitrogen to phosphorus implied by the regressions (i.e. the b values) are 10.2, 25.1, and 34.5 for harvests 2, 3, and 4 respectively. These ratios are of the same order of magnitude as those for purified preparations of nucleoproteins. Greenstein (1944) gives analytical values for mammalian liver nucleoproteins, for which the nitrogen-phosphorus ratio ranges from 17 to 20. Bawden (1943) also gives data for plant viruses from which ratios ranging from 4 to 40 may be calculated; for the greater number, however, they approximate to 30. More recently Best (1948), working with tobacco mosaic virus, presented data for which the nitrogen-phosphorus ratios ranged from 30 to 33. The ratios derived from the regressions admittedly have a rather wide range, but it is very unlikely that the content of protein other than nucleoprotein would be entirely unaffected by treatment, as is assumed by this use of the regressions.

Because of the small amounts present in the tissues, the nucleic-acid phosphorus data for harvests 5, 6, and 7 are less reliable than those already discussed. The data for all three fractions may also be expressed as percentages of the total phosphorus present, and in Figure 5 this has been done for the alcohol-soluble and nucleic-acid phosphorus values for the oat experiment. In general, treatment effects are in the same direction for these two fractions, so that effects on residual phosphorus would be the reverse of these.

The values for alcohol-soluble phosphorus present a simple picture, with $P_2 > P_1 > P_3$ at all harvests. With nucleic-acid phosphorus the same order holds for harvest 3-6, but the percentages with P_1 are the greatest at harvests 2 and 7.

With P_1 , a reduction in the concentration of residual phosphorus is associated with an accelerated hydrolysis of more complex forms (see below), and the maintenance of relatively low partition values for these forms. With P_3 , on the other hand, the concentration of residual phosphorus rises to very high values at first and is associated with retardation of nucleic-acid synthesis; after this phase, the partition values for nucleic-acid and alcohol-soluble phosphorus increase somewhat, and then decline more or less continuously until the final harvest. In late senescence, after harvest 6, when phosphorus is being exported from the leaves, the partition values indicate an accelerated hydrolysis of phosphatides and a relative stability of nucleic acids (especially in P_1).

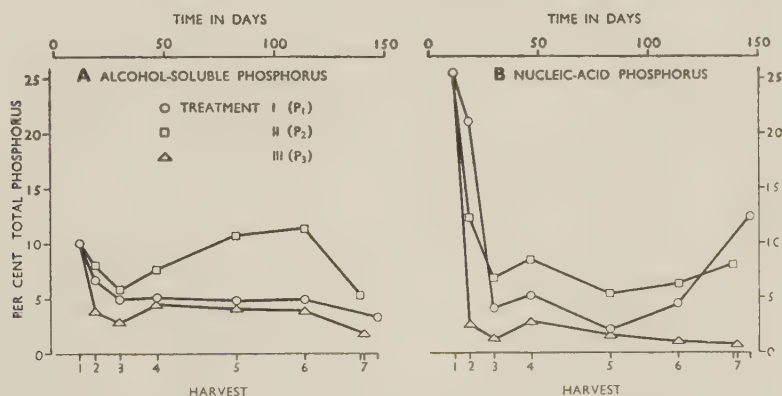


Fig. 5.—Alcohol-soluble and nucleic-acid phosphorus as percentage of total phosphorus in the leaves of oat plants.

The concept of a *partition index* for mineral elements in leaves was used by Phillis and Mason (1939, 1942b) and Mason and Phillis (1943), and was defined as the insoluble fraction of the element expressed as a percentage of the total amount in the leaf. For phosphorus, the insoluble fraction was that which was not pressed out with the sap, and was presumed to contain only “phospho-proteins and phospho-lipoids.” If this assumption was sound, then the percentages of Figure 5 are components of the *partition indices* for oat leaves at seven stages of growth. Only at harvest 2, however, is there any suggestion of a linear correlation (negative) of the index with residual-phosphorus content; the *indices* are considerably lower with P_1 than with P_2 for harvests 3-6 inclusive, and, on the average, *indices* are much smaller for oats than for cotton. It is possible that compounds such as hexose phosphoric esters and adenyl pyrophosphate, which are less likely to be “sap-soluble” than is inorganic phosphorus, may have contributed materially to the “insoluble” fraction of Phillis and Mason. Furthermore, this contribution would increase with increasing phosphorus deficiency, so that a large proportion of the “residual” phosphorus in P_1 would be organically bound. This calls for experimental confirmation, and the whole question of the effects of age and of

phosphorus supply upon the partition of leaf phosphorus could well be elucidated using structurally uniform material and an experimental design similar to that of Walkley and Petrie (1941) for studying the relation between proteins and amino acids in a specified leaf.

(f) *The Initial Growth Depression with High Phosphorus Supply*

The absolute data for nucleic-acid phosphorus indicate that the synthesis of nucleic-acid was immediately retarded with P_3 and to a relatively greater extent than was leaf protein. These further facts necessitate a revision of statements previously made (Williams 1938, p. 77) with respect to the growth depression with high phosphorus supply. It was then established that treatment effects on protein nitrogen preceded those on water content, and, in the absence of any effect on soluble-nitrogen content, it was suggested that the detrimental effect of P_3 on protein synthesis and growth was through an effect on nitrogen intake rather than a direct effect on protein synthesis.

The rate of intake of nitrogen per day was certainly lower with P_3 than with P_2 (Table 2), but the rate per unit weight of root was at first unaffected by treatment and then became greater with P_3 for harvest-interval 3-4 (Fig. 2B). These effects have been examined in an earlier section, and they would seem primarily to be functions of the demand for nitrogen set up by the growth of the various plant parts and by differences in the growth rates of these parts relative to each other.

It now seems that synthesis of nucleoproteins is greatly retarded by high, perhaps excessive, phosphorus supply; this is at once in accord with a reduction in meristematic activity, a check in the rate of dry weight increase, and a reduction in intake of nutrients supplied in constant amount. Any attempt to seek cause-effect sequences between such attributes (structural as well as functional), however, is likely to lead to false emphasis, as in the writer's initial interpretation of the present case. Nevertheless, the retardation of nucleic-acid synthesis is more likely to precede the established effect on nitrogen intake than vice versa.

(g) *Phosphorus Metabolism in Phosphorus-Deficient Leaves*

With adequate (P_2) and excessive (P_3) supplies of phosphorus, the transition from the stage of dependence of the seedling upon seed reserves of phosphorus is rapid, but with deficient (P_1) supply it is slow, and the data warrant detailed examination (Fig. 6).

During harvest-interval 1-2, the dry weight of the leaves increased nearly fourfold, and that of the roots more than twofold. Much of the total increment may be attributed to carbon assimilation by the leaves, but the decrease in the weight of the "stem" fraction (which includes the residue of the grain) indicates continued translocation of materials from the endosperm. Over the same period, no phosphorus was absorbed from the medium, but 0.03 mg. per plant was distributed from the "stem" to the leaves and roots; the leaves obtaining twice as much as the roots.

During the next harvest-interval (2-3), the dry weight of the plant was more than doubled, that of the leaves was doubled, that of the roots was trebled, and that of the "stems" was increased by 50 per cent. At the same time, however, the

absolute-phosphorus contents of the leaves and "stems" remained stationary, and the whole of the phosphorus intake from the medium was retained by the roots. This fact has an important bearing on the effect of phosphorus treatment on root weight ratio, and will be discussed further.

Harvest-interval 3-4 was the period over which the rate of phosphorus intake per unit weight of root was at a maximum (Fig. 2A); the leaves, stems, and roots received phosphorus at rates of 1.37, 2.45, and 1.31 mg. per g. increase in dry weight respectively, and all parts grew rapidly.

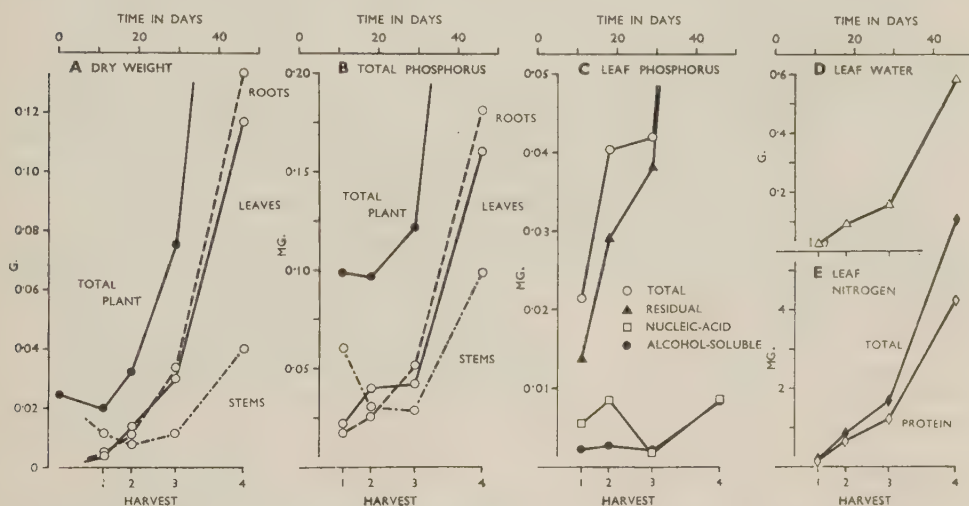


Fig. 6.—Absolute data relating to phosphorus-deficient oat seedlings (for explanation see text).

Thus with P_1 , seed reserves of phosphorus were virtually exhausted 18 days after sowing, and absolute-leaf-phosphorus content was unchanged 11 days later. Leaf growth did not cease, however, as is shown by the increases in dry weight, protein, and water, and there were drastic changes in the partitioning of leaf phosphorus between the three fractions estimated. Thus, during this period (interval 2-3), nucleic-acid phosphorus was reduced to one-fifth of its initial amount; there was a slight decrease in the weight of alcohol-soluble phosphorus; and there was a 30 per cent. increase in residual phosphorus. When the fact of continued growth, with the production of new cells, tissues, and leaves, is taken into account, it will be seen that the hydrolyses here indicated may have been localized in the earlier-formed leaf-cells and tissues. On this basis, too, the resumption of net synthesis indicated by the data (interval 3-4) would primarily be a function of juvenile leaf growth at that time. There was, however, a retardation of total leaf growth between harvests 2 and 3, as is evident from the forms of the curves for dry weight, protein-nitrogen, and water (Figs. 6A, 6E, and 6D). It is tempting to speculate concerning the roles of ribonucleic and deoxyribonucleic acids in this sequence of net synthesis, hydrolysis, and synthesis of nucleic-acid phosphorus, but such speculation would scarcely be profitable since it is believed that the data refer only to total nucleic acid. Reference may

be made to Mirsky (1943) for a review of the literature concerning the localization of the two acids in the nucleus and the cytoplasm.

(h) Root Weight Ratios

This aspect of phosphorus nutrition was discussed by Williams (1936, p. 176) who found that the bulk of the experimental evidence indicated a depression in the root weight ratio with increasing phosphorus supply. Since the leaf and stem weight ratios were affected in a more variable manner, it was inferred that the depression could not be due to a positive stimulation of the growth of leaves or stems alone. This inference gains support from the data presented above in the section on phosphorus metabolism in phosphorus-deficient leaves. With P_1 , the whole of the phosphorus intake for interval 2-3 was retained by the roots, and there was a concurrent stimulation of root growth relative to leaf growth (see Fig. 6): root weight became greater than leaf weight at harvest 3 and remained so for several weeks. The effect of treatment is well shown by a comparison of the relative growth rates of the leaves, R_L , with those of the roots, R_R , as these are affected by stage of growth and treatment (Table 7). For harvest-interval 2-3, R_R is greater than R_L for P_1 , but the reverse is the case for P_2 and P_3 : for interval 3-4, these rates are equal for P_1 , but R_L remains the greater for P_2 and P_3 .

TABLE 7
RELATIVE GROWTH RATE (G. PER G. PER DAY) OF THE LEAVES,* R_L , AND OF THE ROOTS, R_R , OF OATS

Harvest-Interval	P_1		P_2		P_3	
	R_L	R_R	R_L	R_R	R_L	R_R
1-2	0.190	0.123	0.201	0.108	0.211	0.101
2-3	0.072	0.100	0.130	0.118	0.119	0.081
3-4	0.080	0.081	0.138	0.117	0.139	0.132
4-5	0.057	0.047	0.064	0.069	0.067	0.081

* Leaf separations were made by cutting at the ligule, but with the youngest leaf of each axis only that portion was taken which had protruded from the sheath of its predecessor. This procedure may have introduced considerable error in the leaf and "stem" weights at harvest 1 (common to all treatments), but the error would be of rapidly diminishing significance for later harvests. Values of R_L for interval 1-2 were thus arbitrarily increased, though still reasonably comparable as between treatments. The relative shoot growth rates were 0.049, 0.062, and 0.070 g. per g. per day for P_1 , P_2 , and P_3 respectively.

These facts indicate that at least one factor contributing to an increased root weight ratio with phosphorus deficiency may be the fixation of a greater proportion of the absorbed phosphorus, so that relatively little is available for shoot growth: the roots, being nearer the supply, benefit at the expense of other parts of the plant. The stimulation of root growth relative to leaf growth, however, implies a diversion of a greater proportion of carbohydrate from the shoots, so that carbohydrate supply would set the limit to this tendency.

In his studies of root growth in Lemna, White (1937, 1938) interprets his results in terms of carbohydrate-nitrogen balance in the fronds, and he regards this interpretation as a particular case of a general phenomenon with respect

to the control of root development. This may be accepted for his own experiments, and for those experiments cited (White 1937, p. 652) in which carbohydrate level was obviously affected by treatment, but in those experiments in which the root weight ratio was increased by nitrogen deficiency, it seems probable that the roots, being nearer to the limited supply of nitrogen, may have benefited at the expense of other plant parts.

The difference between the two mechanisms lies in the fact that the roles of the roots and of the shoots respectively are emphasized — perhaps over-emphasized. The relative importance of the mineral nutrient or the carbohydrate level tends also to be overstated in these attempts to seek cause-effect sequences. Once again, the remedy may lie in the restatement of the problem of organic growth, and a departure from the strictly analytic procedures which have been applied in the past.

(i) *Nucleo-Cytoplasmic Ratios*

Robertson and Dawbarn (1929) determined nucleic-acid nitrogen and coagulated nitrogen contents of various organs of the new-born lamb and the adult sheep, and they found that nucleo-cytoplasmic ratios based on these values fell with age in all organs examined. They also concluded that there must be an essential interdependence of nucleic-acid and protein synthesis in animal tissues. Huelin (1929) used the same methods (Robertson 1929) with wheat plants at three stages of growth, and found also that the ratio decreased rapidly with time.

The nucleo-cytoplasmic ratios of Table 8 were computed from the nucleic-acid phosphorus and protein nitrogen data of the oat experiment, using the factor 1.693 to convert nucleic-acid phosphorus to nucleic-acid nitrogen. The ratios cover a much wider range of growth stage and treatment than those of Huelin for wheat. During early growth, the ratios decrease rapidly from a high initial

TABLE 8
NUCLEO-CYTOPLASMIC RATIOS FOR OAT LEAVES ($\times 100$)

Harvest	1	2	3	4	5	6	7		
Days from Sowing	11	18	29	46	82	113	138	140	146
P ₁	6.44	2.25	0.24	0.34	0.12	0.25	—	—	0.61
P ₂	6.44	3.32	1.69	1.13	0.91	0.76	0.66	—	—
P ₃	6.44	1.83	0.99	1.08	1.39	0.88	—	0.72	—

value common to all treatments; only with P₂, however, does this trend continue on to full maturity. The initial decreases are greater with P₁ and P₃ than they are with P₂, but subsequent trends are more erratic. Treatment effects are largely a reflection of the effects on nucleic-acid content.

These nucleo-cytoplasmic ratios were examined in relation to the growth data of the experiment, and, in particular, to the relative leaf growth rates. No obvious correlations were revealed.

(j) *Total, Alcohol-Soluble, and Nucleic-Acid Phosphorus in the Leaves of Phalaris tuberosa L.*

The growth data for the experiment with *Phalaris* have been presented elsewhere (Williams 1946), and the phosphorus analyses of the leaf material were conducted in order to determine the effect of increased phosphorus supply at two levels of nitrogen. The experiment covered the period of rapid vegetative growth prior to stem elongation, and leaf dry weights were increasing throughout the period of investigation (day 69 to day 139). Relative and absolute values for total, alcohol-soluble, and nucleic-acid phosphorus, and for protein nitrogen are presented in Figure 7.

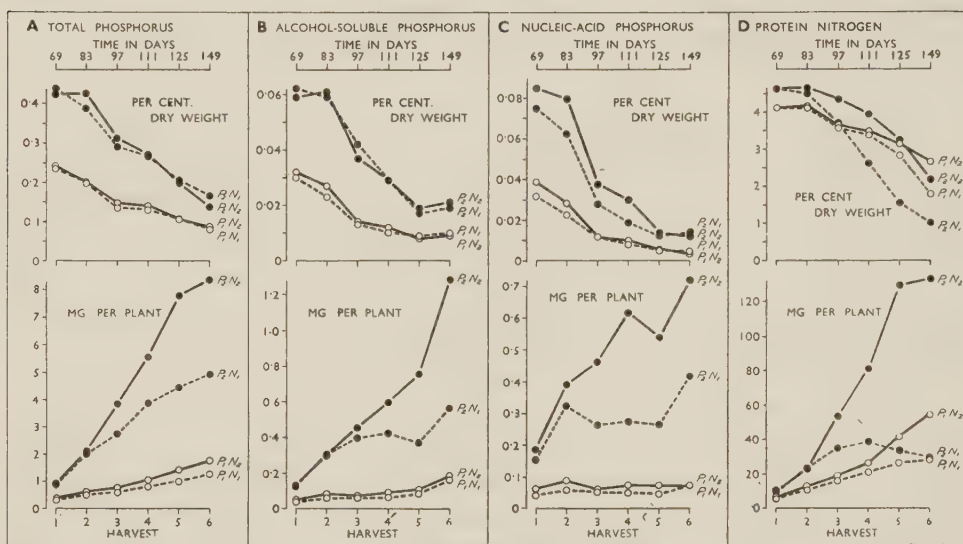


Fig. 7.—Relative and absolute amounts of total, alcohol-soluble, and nucleic-acid phosphorus, and of protein nitrogen in the leaves of *Phalaris*.

The sixfold phosphorus treatment, P_2 , did little more than double the relative phosphorus content at any given harvest, and there was no clear-cut effect of the increased supply of nitrogen. The time trends in, and treatment effects on, the relative contents of alcohol-soluble phosphorus present a similar picture, except for increases with all treatments from harvest 5 to harvest 6. With nucleic-acid phosphorus, however, the time trends are more pronounced, and there are quite marked increases with N_2 . Thus, P_1N_2 exceeds P_1N_1 at harvests 1 and 2, and P_2N_2 exceeds P_2N_1 for harvests 1 to 4 inclusive. As in the oat experiment, similarities of treatment effect on nucleic-acid phosphorus and protein-nitrogen contents are evident for early growth stages (harvests 1 and 2), and the ratio of nitrogen to phosphorus derived from a regression (*vide supra*) is approximately 11.5.

Although leaf weights were still increasing rapidly between harvests 5 and 6, there is a suggestion that the absolute phosphorus contents are approaching their maxima with P_2N_1 and P_2N_2 . Protein nitrogen is at a maximum at harvest

4 with P_2N_1 , and is probably so at harvest 6 with P_2N_2 . Time trends in the absolute contents of alcohol-soluble and nucleic-acid phosphorus are somewhat erratic, though closer inspection, especially of the latter, reveals fairly consistent departures from the general trend. Thus, at harvests 2, 4, and 6 these departures tend to be positive, and at harvests 1, 3, and 5 they tend to be negative. Furthermore, these departures tend to be the reverse of those in protein nitrogen, in total phosphorus, and in relative leaf growth rate. When the growth rate is relatively high, the net synthesis of protein and the net intake of phosphorus are high, but the net increment in nucleic-acid phosphorus is small or even negative. Quite tentatively, it is suggested that rapid development of new leaf and stem tissues sets up a demand for phosphorus such that hydrolysis of nucleic acid, and to a lesser extent of phosphatides is hastened in the earlier-formed leaves, the whole being governed by the form of the steady-state relation between these constituents and their soluble phosphoric precursors.

The partition values for this experiment are given in Table 9. Those for alcohol-soluble phosphorus show no features of special significance, but those for

TABLE 9
ALCOHOL-SOLUBLE AND NUCLEIC-ACID PHOSPHORUS EXPRESSED AS PERCENTAGES OF
TOTAL PHOSPHORUS IN THE LEAVES OF PHALARIS TUBEROSA L.

Har- vest	Days from Sowing	Alcohol-Soluble P				Nucleic-Acid P			
		P_1N_1	P_1N_2	P_2N_1	P_2N_2	P_1N_1	P_1N_2	P_2N_1	P_2N_2
1	69	12.7	13.0	14.1	13.9	13.5	16.1	17.1	20.0
2	83	11.5	13.4	15.2	14.4	11.3	14.1	16.0	18.6
3	97	9.7	9.8	14.3	11.9	8.6	8.0	9.6	12.0
4	111	7.9	8.6	11.1	10.6	6.0	6.9	7.1	11.0
5	125	8.1	7.0	8.2	9.5	4.5	5.1	5.9	6.9
6	149	12.3	10.5	11.2	15.0	5.8	4.0	8.4	8.6

nucleic-acid exhibit the following characteristics: a general decline from a mean of 17 per cent. at harvest 1 to one of 6 per cent. at harvest 5; consistent increases with high phosphorus supply, these being more pronounced at N_2 than at N_1 ; and increases with high nitrogen supply, these being maintained until harvest 5 with P_2 .

The nucleo-cytoplasmic ratios were also examined, but, as in the experiment with oats, these revealed no obvious correlation with relative leaf growth rate.

IV. GENERAL DISCUSSION

(a) *Phosphorus Fractionation in Plant Tissues*

A full review of the literature covering all aspects of phosphorus nutrition touched upon in this paper would be quite inappropriate, but a review of selected contributions to our knowledge of phosphorus fractionation within the plant may help to focus attention on a neglected field of plant-biochemical and physiological research. Available methods for the estimation of phosphatides, phosphoric esters, nucleic acids, and of nucleic-acid derivatives (such as adenylyl pyrophosphate and certain enzymes) leave much to be desired, and lack of critical methods

greatly reduces the value of comparisons between existing sets of data. Procedures more recently elaborated by Arney (1939) and by Albaum and Umbreit (1943) would seem to be an improvement on those previously used, but they are still far too empirical.

Webster (1928) studied the phosphorus distribution in 11 types of grain. Phytin phosphorus tended to be the dominant form, and both inorganic and phosphatide phosphorus were present in very small proportions. The data give no satisfactory index of nucleic-acid content. Using more critical methods, Javillier and Colin (1933) give the following figures:

		Wheat Germ	Lentil Powder
As % of Total Phosphorus	Inorganic P	21	26
	Phytin P	42	51
	Phosphatide P	9	12
	Nucleic-acid P	28	11

Webster and Dalbom (1930) give data for field-grown mung beans at ten stages of growth. In all vegetative parts, organic-phosphorus content fell rapidly with time whereas inorganic-phosphorus content commenced at a low value and remained relatively constant. Expressed as a percentage of the total phosphorus, inorganic phosphorus increased from 10 to nearly 30 per cent. in the leaves; in the stems, it rose from 14 to 88 per cent., but fell again to 55 per cent. at maturity; whereas in the pods, inorganic phosphorus constituted only 14 per cent. of the total phosphorus present. Only traces of lipid phosphorus (alcohol-ether extraction) were found in any plant parts. The high proportion of inorganic phosphorus in the stems at the time of rapid seed development suggests that phosphorus is translocated in this form. In the following year the same workers compared mung beans grown with and without superphosphate (600 lb. per acre), and at two stages of growth. Treatment had little effect upon the amount of growth, and the absence of any considerable effects upon the total or inorganic-phosphorus contents indicates that little extra phosphorus was taken into the plants.

Knowles and Watkin (1932), using field-grown wheat, determined phosphatide, phytin, and "inorganic" phosphorus, firstly, in the total shoots and later in ears and straw separately (9 growth stages in all). Their work is open to criticism in that they air-dried their material, thus giving greater opportunity for hydrolysis and other changes during drying. Furthermore, their "inorganic" phosphorus was obtained by difference and presumably includes the phosphorus of nucleic acid and of the simpler phosphoric esters. Absolute phosphorus per tiller increased with time in a manner similar to that of the shoots of oat plants receiving P_2 in the present experiments (see Fig. 1); almost 85 per cent. of this was located in the ear at final harvest. Alcohol-soluble and "inorganic" phosphorus were at their maxima soon after the appearance of the ears. Phytin phosphorus was also at a maximum in the straw at this time, but continued to form in the ear up to final harvest. The disappearance of "inorganic" phosphorus from the straw is difficult to reconcile with other data, and throws doubt upon the values given for phytin phosphorus in the straw (95 per cent. of the phosphorus present). The

phytin content of the ears (50 per cent. of the total) agrees substantially with other work. DeTurk, Holbert, and Howk (1933) found no phytin in the vegetative parts of maize, but it appeared in the seeds two weeks after pollination.

DeTurk, Holbert, and Howk (*loc. cit.*) and Burgevin and Guyon (1933) studied phosphorus fractionation in yellow dent corn and spring barley respectively. Their phosphorus fractions were obtained by successive extraction with absolute alcohol, acid alcohol, and dilute hydrochloric acid; these gave crude indices of phosphatide, inorganic, and esterified phosphorus (including phytin), and the residual phosphorus was an index of nucleic-acid phosphorus. DeTurk, Holbert, and Howk demonstrated the need for rapid drying of their material before extraction; that of Burgevin and Guyon was air-dried. The time trends in the data of DeTurk, Holbert, and Howk for the shoots (excluding ears when present) of two first-generation crosses of corn grown on untreated and phosphorus-treated soil may be summarized by the following mean partition values (per cent. total phosphorus) for the five sampling occasions:

	July 6	July 18	August 1	August 11	August 22
Inorganic P	27	34	35	39	33
Phosphoric-ester P	47	42	32	27	28
Phosphatide P	14	11	14	12	14
Nucleic-acid P	12	13	19	22	25

On the dry-weight basis, the phosphoric ester content fell rapidly with time, whereas the nucleic-acid content remained relatively constant. The two first-generation crosses differed markedly in their response to phosphorus: where the yield was increased, there were also increases in inorganic and nucleic-acid phosphorus, especially for the early sampling occasions; but in the case where there was a negligible increase in yield, the only consistent effect on the phosphorus fractions was a depression in the nucleic-acid content after the first harvest. Burgevin and Guyon found that, prior to earing, phosphorus treatment increased both the relative and absolute amounts of all four of their fractions in the shoots of barley. Inorganic phosphorus had the largest partition value and this value was slightly increased by treatment. Phosphorus intake by the shoots continued to the end of the experiment, and at maturity the dominant fraction was that soluble in dilute hydrochloric acid: at this stage this fraction would occur largely as phytin in the grain. Phosphatide phosphorus at no time exceeded 10 per cent. of the total phosphorus present.

While Strebeyko (1934) presents values for phosphorus fractions in the shoots of oats for only the first two of his five sampling occasions, his experiment has certain features in common with the present experiment with oats. There were five levels of supply of phosphorus* and his dry-weight, phosphorus, and nitrogen data include separate values for grain and straw for harvests 4 and 5. Some analyses for sulphur, potash, and protein nitrogen are given for early stages. Growth responses were considerable up to treatments 3, 4, and 5. The data confirm the following points: (i) phosphorus contents of the straw fall

* Treatments were 0, 0.02, 0.05, 0.10, and 0.20 g. P_2O_5 per pot for treatments 1-5 respectively; treatment 5 would approximate to P_2 of the present experiment.

ultimately to the same level with all but the highest supplies of phosphorus; (ii) with phosphorus deficiency, a greater percentage of the total intake is obtained during the second half of the growth period; (iii) for early growth stages, the nitrogen content of vegetative parts is at first increased, but then decreased by increasing phosphorus supply; (iv) for later growth stages, the nitrogen content of all parts is decreased by increasing phosphorus supply; and (v) the ratio of protein to total nitrogen tends to be decreased by phosphorus deficiency.

Strebeiko's phosphorus fractionation data may be restated in the form of partition values, thus:

Treatment	Day 35					Day 45				
	1	2	3	4	5	1	2	3	4	5
Mineral P	45	38	63	62	61	29	29	38	40	45
Phytin P	25	17	9	10	10	25	19	20	19	18
Insoluble Organic P	29	45	28	28	29	46	52	42	40	37

It is not clear where the phosphoric esters were included in these analyses, and the "insoluble organic" fraction would appear to include both phosphatides and nucleic acids. On a dry-weight basis phytin phosphorus is the only fraction which does not increase markedly with increasing phosphorus supply. In this, as in a later set of data (Strebeiko 1939), the partition values for insoluble organic-phosphorus tend to maxima with supplies in the range 0.0 to 0.04 g. P_2O_5 per pot. This is in accord with the pronounced maxima with P_2 in the partition values for alcohol-soluble and nucleic-acid phosphorus in the present experiment.

(b) *The Interpretation of Growth Data*

That growth in plants should be thought of as a complex and closely integrated process can scarcely be regarded as a novel idea, but few attempts have been made to develop its implications. Scientific method must, of necessity, abstract from this complexity, but biological interpretation should not end at the physico-chemical level of organization (Woodger 1929, Chapter VI). In this connection, research on plant hormones has made considerable contributions, for, as Went (1940) says, "the hormone concept transcends the differentiation of the organism into cells; hormones integrate cells into an organism."

In the wider study of plant growth, however, where irreversible increase in volume is not the only attribute under consideration, the complexity is such that much might be gained by concentration on the higher rather than the lower relevant levels of organization. This has, in effect, been suggested by Bald (1946), who proposes "a principle of competition for the available metabolites between the various organs of the plant" as the basis for a general plan of growth for the potato plant. Similarly, when some specific function, such as the intake of a nutrient by the plant as a whole, is under discussion, the same principle of competition may be applied with advantage, without necessarily enquiring into the details of the mechanisms which set up the demand for available metabolites. Here again, the principle of competition between plant parts is no new idea (Went 1935), but relatively few sets of data in the literature are sufficiently comprehensive to make its application worth while.

The present paper is one of a series* which has been devoted to the study of certain of the physiological changes that occur in plants during ontogenesis. Stress is laid upon the interrelations of the attributes measured, and the ultimate task in the study of the growth is thought of as the elucidation of the integrated pattern which is characteristic of the living organism.

Experimental variation of this pattern has been achieved by varying the nutrient and water supplies, and by preventing the development of the inflorescence. The attributes measured include the dry-weights of the plant and its parts, the leaf area, and leaf respiration, the absolute and relative contents of water, nitrogen, protein-nitrogen, and phosphorus. To these are now added the phosphorus fractions considered above. Consideration has been given to the interpretation of the changes in dry-weight, leaf area and nutrient content of the whole plant and its composite parts (see especially Watson and Petrie 1940, pp. 323-35). This interpretation is tentatively made in terms of the processes of cell division, cell extension, accumulation of reserves, and differentiation, and of the known determinants of these processes. The interpretations thus tend to be rather too abstract, for little is yet known of the relations between these processes. However, in the case of discussions relating to the redistribution of nitrogen within the plant (Williams 1938, p. 78; Watson and Petrie 1940, p. 332), the idea of active competition between the plant parts is developed at some length. Use is made of units which are characteristic of the higher levels of organization in the living plant, and it is evident that the meristematic tissues are largely involved in the control of the movement of carbohydrate reserves and mineral nutrients within the plant. This is clearly in accord with the view of Hoagland, stated in the introduction to this paper, that the plant should be envisaged as an integrated organism; it implies that explanation should be sought at the biological as well as at the physico-chemical level of organization (Woodger loc. cit.).

An attempt has been made to apply these principles to the data under discussion, with the result that rates of intake of phosphorus were seen to be determined more by the internal factors of demand than by the external factor of supply. Then, too, specific effects of the variation of phosphorus supply on plant establishment, ratios of roots to shoots, and the redistribution of phosphorus within the plant are readily understood in terms of the competitive demand for nutrients by meristematic tissues.

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* *Gramineous plants*: Ballard and Petrie (1936); Petrie (1937); Petrie and Williams (1938); Williams (1936, 1938, 1939, 1946). *Tobacco*: Petrie and Arthur (1943); Petrie, Watson, and Ward (1939); Ward and Petrie (1940); Watson (1939); Watson and Petrie (1940). *Flax*: Tiver (1942); Tiver and Williams (1943).

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VI. REFERENCES

- ALBAUM, H. G., and UMBREIT, W. W. (1943).—*Amer. J. Bot.* **30**: 553.
 ARNEY, S. E. (1939).—*Biochem. J.* **33**: 1078.
 ARNON, D. I., STOUT, P. R., and SIPOS, F. (1940).—*Amer. J. Bot.* **27**: 791.
 BALD, J. G. (1946).—*Emp. J. Exp. Agric.* **14**: 43.
 BALLARD, L. A. T., and PETRIE, A. H. K. (1936).—*Aust. J. Exp. Biol. Med. Sci.* **14**: 135.
 BAWDEN, F. C. (1943).—“Plant Viruses and Virus Diseases.” (Chronica Botanica Co.: U.S.A.)
 BEST, R. J. (1948).—*Aust. J. Exp. Biol. Med. Sci.* **26**: 65.
 BIDDULPH, O. (1941).—*Amer. J. Bot.* **28**: 348.
 BURGEVIN, H., and GUYON, G. (1933).—*Ann. Agron.* **3**: 663.
 CROWTHER, F. (1934).—*Ann. Bot.* **48**: 877.
 DETURK, E. E., HOLBERT, J. R., and HOWK, B. W. (1933).—*J. Agric. Res.* **46**: 121.
 DUNLAP, A. A. (1939).—*Amer. J. Bot.* **26**: 15.
 FRANK, L. K. (1935).—*Philos. Sci.* **2**: 210.
 GREENSTEIN, J. P. (1944).—*Advances Protein Chem.* **1**: 209.
 GREGORY, F. G., and RICHARDS, F. J. (1929).—*Ann. Bot.* **43**: 119.
 HALPERN, G. R. (1945).—*Nature* **155**: 110.
 HOAGLAND, D. R. (1944).—“Inorganic Nutrition of Plants.” (Chronica Botanica Co.: U.S.A.)
 HUELIN, F. E. (1929).—*Aust. J. Exp. Biol. Med. Sci.* **6**: 59.
 JAVILLIER, M., and ALLAIRE, H. (1931).—*Bull. Soc. Chim. Biol.* **13**: 678.
 JAVILLIER, M., and COLIN, Y. (1933).—*Ibid.* **15**: 1552.
 JORDAN, R. C., and CHIBNALL, A. C. (1933).—*Ann. Bot.* **47**: 163.
 KNOWLES, F., and WATKIN, J. E. (1932).—*J. Agric. Sci.* **22**: 755.
 KREYZI, R. (1932).—*Z. PflErnähr. Düng. A* **25**: 156.
 MASON, T. G., and PHILLIS, E. (1943).—*Ann. Bot. (N.S.)* **7**: 399.
 MIRSKY, A. E. (1943).—*Advances Enzymol.* **3**: 1.
 PETRIE, A. H. K. (1934).—*Aust. J. Exp. Biol. Med. Sci.* **12**: 99.
 PETRIE, A. H. K. (1937).—*Ibid.* **15**: 385.
 PETRIE, A. H. K., WATSON, R., and WARD, E. D. (1939).—*Ibid.* **17**: 93.
 PETRIE, A. H. K., and ARTHUR, J. I. (1943).—*Ibid.* **21**: 191.
 PETRIE, A. H. K., and WILLIAMS, R. F. (1938).—*Ibid.* **16**: 347.
 PHILLIS, E., and MASON, T. G. (1939).—*Ann. Bot. (N.S.)* **3**: 569.
 PHILLIS, E., and MASON, T. G. (1942a).—*Ibid.* **6**: 437.
 PHILLIS, E., and MASON, T. G. (1942b).—*Ibid.* **6**: 469.
 REID, M. E., (1941).—*Amer. J. Bot.* **28**: 45.
 REWALD, B. (1936).—*Chem. & Ind.* **14**: 1002.
 REWALD, B. (1937a).—*J. Soc. Chem. Ind.* **56**: 77.
 REWALD, B. (1937b).—*Enzymologia* **3**: 10.
 ROBERTSON, T. B. (1929).—*Aust. J. Exp. Biol. Med. Sci.* **6**: 33.
 ROBERTSON, T. B., and DAWBARN, M. C. (1929).—*Ibid.* **6**: 261.
 STREBEYKO, P. (1934).—*Acta Soc. Bot. Polon.* **11**: 213.
 STREBEYKO, P. (1939).—*Planta* **29**: 477.
 TIVER, N. S. (1942).—*Aust. J. Exp. Biol. Med. Sci.* **20**: 149.
 TIVER, N. S., and WILLIAMS, R. F. (1943).—*Ibid.* **21**: 201.
 WALKLEY, J. (1940).—*New Phytol.* **39**: 362.

- WALKLEY, J., and PETRIE, A. H. K. (1941).—*Ann. Bot. (N.S.)* **5**: 661.
- WARD, E. D., and PETRIE, A. H. K. (1940).—*Aust. J. Exp. Biol. Med. Sci.* **18**: 21.
- WATSON, R. (1939).—*Ibid.* **17**: 241.
- WATSON, R., and PETRIE, A. H. K. (1940).—*Ibid.* **18**: 313.
- WEBSTER, J. E. (1928).—*J. Agric. Res.* **37**: 123.
- WEBSTER, J. E., and DALBOM, C. (1930).—*Ibid.* **41**: 819.
- WENT, F. W. (1935).—*Proc. Acad. Sci. Amst.* **38**: 752.
- WENT, F. W. (1940).—From Baitzell, G. A., "Science in Progress."
- WHITE, H. L. (1937).—*Ann. Bot. (N.S.)* **1**: 649.
- WHITE, H. L. (1938).—*Ibid.* **2**: 911.
- WILLIAMS, R. F. (1936).—*Aust. J. Exp. Biol. Med. Sci.* **14**: 165.
- WILLIAMS, R. F. (1938).—*Ibid.* **16**: 65.
- WILLIAMS, R. F. (1939).—*Ibid.* **17**: 123.
- WILLIAMS, R. F. (1945).—*Ibid.* **23**: 213.
- WILLIAMS, R. F. (1946).—*Ann. Bot. (N.S.)* **10**: 41.
- WOODGER, J. H. (1929).—"Biological Principles." (Kegan Paul: London & New York.)
- ZINZADZE, C. (1931).—*Proc. Internat. Soc. Soil. Sci.* **6**: 95.
- ZINZADZE, C. (1935).—*Industr. Engng. Chem. (Anal. Ed.)* **7**: 227.

NUTRITIONAL FACTORS INVOLVED IN WOOL PRODUCTION BY MERINO SHEEP

I. THE INFLUENCE OF FODDER INTAKE ON THE RATE OF WOOL GROWTH

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Summary

The rate of wool growth (weight of wool produced per unit time), determined in seven sheep subjected to four levels of food intake, increased 400-600 per cent. from the lowest to the highest level of feeding and this change was reflected in both the mean length and in the mean diameter of the fibres, the relationship between these variables being characteristic of the individual sheep. The fibre diameter distributions were also affected, the individual fibres responding up to the limit of their capacities in proportion to their cross-sectional areas.

The nutritional factors involved in these changes were assessed from complete energy and nitrogen balances and the findings were interpreted in light of existing knowledge of the physiology of amino acid utilization.

From the amino acid constitutions of the proteins in the diet and in the main products of these transactions (wool and flesh) it was estimated that the cystine and methionine content of the protein of diet 541 would limit to 27 per cent. the efficiency of its conversion to wool. Under the conditions of the experiment the efficiency of the utilization of the sulphur-containing amino acids for wool production was at its highest, 47 per cent., when the best wool producer was in strongly positive energy balance, and at its lowest, 14 per cent., when the poorest producers were close to energy equilibrium.

From these observations it was concluded that a Merino sheep grazing on natural pastures would rarely, if ever, exhibit its full wool producing propensity and, as a corollary, that the rate of wool production by a grazing sheep would vary considerably with the seasonal changes in its nutritional environment, and that this would be reflected in its wool staple. Experimental evidence to support this conclusion is submitted.

I. INTRODUCTION

The ultimate nutritional conditions that determine the rate of wool growth are, with little doubt, the concentration and composition of the assemblage of amino acids in the tissue-fluids which surround the wool follicles. The quota of the amino acids and simple peptides absorbed from the intestine which thus eventually becomes available to serve as a substrate for wool production is influenced by physiological interactions that might be expected, *a priori*, to alter with the nutritional state of the sheep.

The supply of amino acids is determined initially by the quantity and quality of the protein ingested in the fodder, and this, under certain circumstances, may be augmented with amino acids from the digestion products of the protein elaborated from simpler nitrogenous substances by the microflora of the rumen; its fate,

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however, depends mainly on the state of the energy balance of the animal. When the energy requirements are met completely by the oxidation of constituents other than the protein of the ration, the supply of substrate is influenced according to whether the relative rates of absorption and of utilization by synthetic processes allow the concentration of amino acids in the blood stream to rise above the deamination threshold. The quota that escapes deamination serves the organism's demands for amino acids, and, at any level of nitrogen equilibrium, may be depleted by the protein syntheses involved in growth, pregnancy, and lactation – with which processes the proliferating cells in the follicles would have to compete. When the available energy of the ration approximates closely the energy dissipated by the animal, the protein digestion products are drawn on heavily to serve as fuel, and so the removal of amino acids from the systemic circulation for this purpose seriously depletes the substrate. When the animal is frankly in negative energy balance and is drawing on its own tissues to make up the deficit, the factors that determine the amino acid supply to the follicles are influenced by the increased nitrogen turnover arising from the hydrolysis of tissue proteins. Under these circumstances, the supply of substrate may be augmented; if the concentration of protein in the fodder is low and the ration is reduced to a level that is grossly insufficient to meet the energy requirements, the rate of wool growth tends to increase for a period above that which prevailed when the ration was just enough to maintain the animal in energy equilibrium.

These considerations of the intermediary metabolic processes known to be involved in the utilization of amino acids indicate that the nutritional factors which primarily influence wool production are the protein available from the ration, the quality of this protein, and the intake of other dietary constituents which are capable of providing for the energy demands* of the animal. The former variables determine the total substrate of amino acids and its composition, and the latter that portion of it which eventually becomes available for the competing protein syntheses involved in wool production, growth, pregnancy, lactation, etc.

The experiment described below, which was designed originally to study changes in the energy transactions of the sheep at different levels of feeding, afforded an opportunity to estimate the influence of nutrition on wool production under conditions in which there was a precise knowledge of the state of the energy balance. It provided also a means of assessing the magnitude of the processes that compete for the amino acids absorbed from the fodder.

As the composition of the diet in these experiments was constant, both the available energy and available protein altered proportionately when the level of intake was changed.

* It is not feasible to estimate directly the actual amount of energy that is dissipated by the synthetic processes involved in wool production, but from what is known of the thermodynamics of amino acid condensations this should be small. The amount of metabolizable energy which is drawn upon for wool production *per se* is thus unlikely to exceed materially the combustible energy of the wool fibre and of the secretions which accompany it—a quantity which in these experiments was never more than 3 per cent. of the metabolizable energy derived from the rations.

II. EXPERIMENTAL PROCEDURE

The animals that were employed, the experimental regime to which they were subjected, the diet on which they were fed, and the various procedures that were used to determine their nutritional status have been described in a previous paper (Marston 1948). In brief, seven strong-wooled (Anama strain) Merino ewes were fed with a fodder, diet 541 (a mixture of 5 parts of crushed wheat, 4 parts of chaffed lucerne hay, and 1 part of cane molasses, compressed into pellets) at 4 levels which ranged in stages, according to the available energy intake, from approximately $\frac{1}{2}$ maintenance to 2 maintenance. In the following discussion, periods, 1, 2, 3, and 4 refer to the 14 days of the 9th and 10th weeks on the approximate levels of fodder intake $\frac{1}{2}$, 1, $1\frac{1}{2}$, and 2 maintenance, respectively — they do not indicate the order in which the fodder was presented to the animals. Complete energy and nitrogen balance sheets were derived from the analytical data.

The comparative wool growth was determined directly. The wool produced on 15 cm. x 10 cm. areas delineated by tattoo on either shoulder of each individual was collected at fortnightly intervals by shaving; it was treated as previously described (Marston 1935). The total wool production was computed from the dry weight of wool collected, the skin area ($A = 0.117W^{0.59}$ where A = skin area in m.², and W = wt. in kg. of the ewe fasted 72 hr. from maintenance level) and the predetermined ratio of the wool grown per unit area on the patch to that grown over the whole skin area which, for this strain, was close to 1.4.

The mean fibre diameters of the samples were determined by measuring the sharply focused, magnified image (500x) of the dry fibres embedded in cedar oil. In each case, 1,000 fibres were measured at random.

III. RESULTS

(a) *The Rate of Change in the Wool Growth Supervening on an Alteration of the Level of Feeding*

Preliminary experiments indicated that an alteration in the rate of wool growth followed immediately a change in the level of fodder intake, but it was evident that a considerable period elapsed before the rate of wool production became constant. In the main experiment here under discussion, the ewes were fed at each level for a period of 10 weeks, the collections from which the balance data were derived being made over the 14 days of the 9th and 10th weeks of this cycle. The wool was collected from the tattooed areas at fortnightly intervals throughout and the data derived from these consecutive collections proved conclusively that equilibrium between the level of intake of this particular fodder and the rate of wool production became established only after the lapse of 3 months, or more, subsequent to the relatively large changes in the level of feeding that were imposed.

This phenomenon is illustrated in Figure 1 by the performance of two typical cases, No. 572 and No. 547, selected at random from the experimental animals. Prior to the experiment, both had been fed for some months on rations

which ensured a slight positive energy balance, and under these conditions ewe No. 572 produced 1.62 g. wool/150 cm.²/14 days and ewe No. 547, 1.70 g. wool/150 cm.²/14 days on the tattooed areas. At the beginning of the experiment the fodder was changed to diet 541 which was fed at the 2M level to No. 572 and at the $\frac{1}{2}$ M level to No. 547. The rate of wool production of the former increased, and that of the latter decreased, but it is clear from the observations of the wool grown in the subsequent periods (Fig. 1) that in neither case was a steady state rapidly attained. Although the food intake was constant for each 10-week

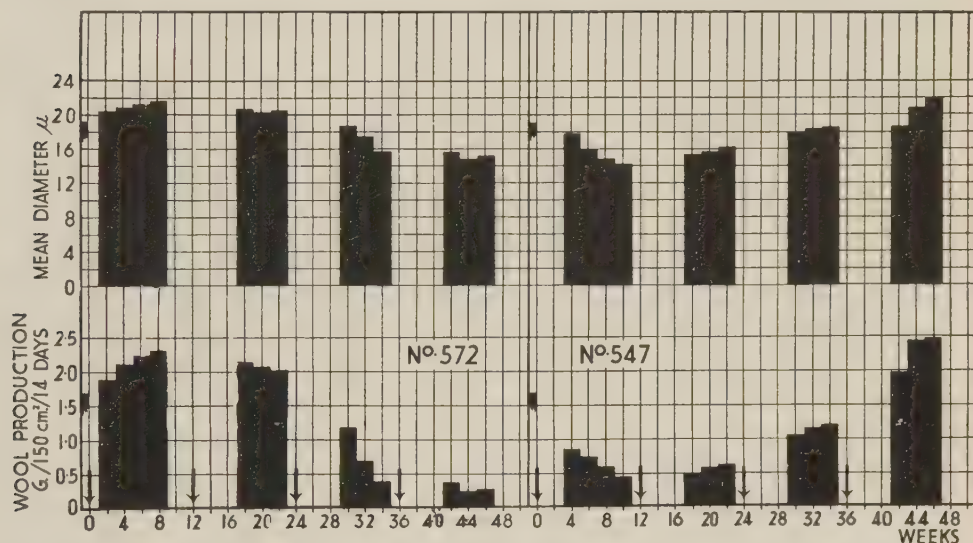


Fig. 1.—The relative slowness in the establishment of an equilibrium between food intake and the rate of wool growth is shown. The fodder was changed at 0 weeks to diet 541 which was fed to No. 572 at 2M level and to No. 547 at $\frac{1}{2}$ M level. The rate of wool growth continued to change for a considerable period after each alteration of fodder intake and tended slowly to approach an asymptote which would imply a state of nutritional equilibrium. This phenomenon supervenes on each alteration of fodder intake level (indicated by arrows). The levels of intake were approximately 2, $1\frac{1}{2}$, 1, and $\frac{1}{2}$ maintenance in respect to the energy available from the ration. The changes for No. 572 were made at intervals of 12 weeks in that order and for No. 547 in the reverse order.

step, the rate of wool growth altered slowly during this period and tended gradually towards the asymptote which would signify that nutritional equilibrium had been established. The observed changes in the rate of wool production which supervened on subsequent alterations of the level of fodder intake, whether the levels of intake were increased or decreased, reflected the same phenomenon.

The nature of the physiological processes responsible for this lag in the establishment of nutritional equilibrium changed according to the level of feeding: at the lowest level, steady depletion of the fat reserves was the major factor which determined the quantity of amino acids drawn upon for fuel, and so the quota that became available for wool production; and at the levels above maintenance, synthetic processes other than wool production were mainly responsible for depletion of the substrate. In neither case was their influence

constant. Nutritional equilibrium was impossible at the lowest level of feeding, and at the higher levels, a steady state could be reached only if the laying down of tissue protein either proceeded at a constant rate, or ceased altogether as it would when the animal had attained its maximum capacity to store protein. The extent to which these factors operated during the collection periods that began 8 weeks subsequent to each change in intake level may be derived from the nitrogen and energy balances discussed below.

(b) *The Relationship between Food Intake and the Rate of Wool Production*

The general phenomenon of the dependence of the rate of wool production upon the level of feeding is obvious from the data set out in Table 1 and Figure 2. These indicate the relative rates of wool growth over the 14 days of the 9th and 10th weeks after each change in the level of food intake; the first three ewes

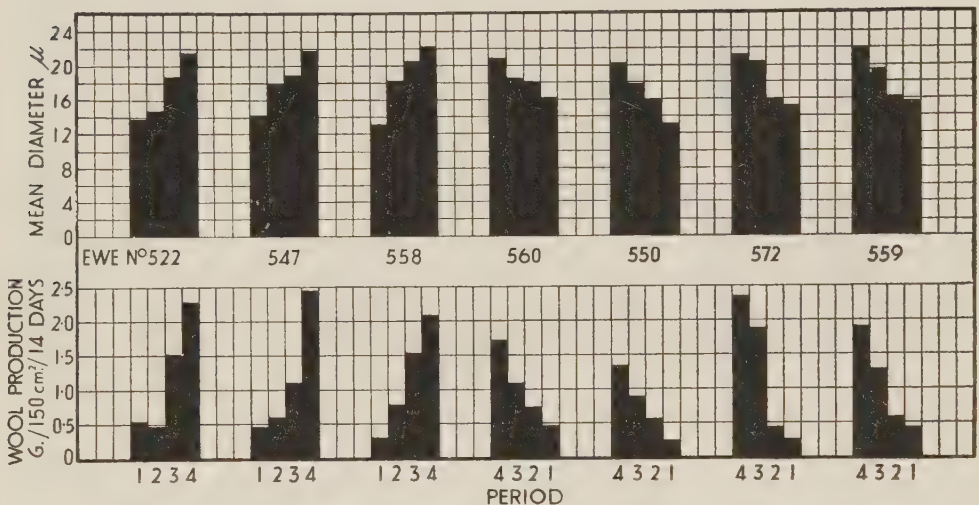


Fig. 2.—The influence of the level of fodder intake on the rate of wool production and mean diameter of the fibres is shown. The available energy intake from diet 541 was approximately $\frac{1}{2}$, 1, $1\frac{1}{2}$, and 2 maintenance, and the available protein intake was approximately 25, 50, 75, and 100 g. per day, for periods 1, 2, 3, and 4 respectively. The levels of feeding were changed in ascending steps for Nos. 522, 547, and 558, and in descending steps for Nos. 560, 550, 572, and 559. The ration was fed for 8 weeks prior to the beginning of each of the periods reported.

were fed in ascending stages and the other four in descending stages. The daily intakes of nitrogen over each of the 10-week intervals and the state of the nitrogen and energy balances during the 14 days of the periods, in which the wools referred to in Table 1 and Figure 2 were collected, are set out in Table 2.

(c) *The Rate of Wool Production in Relationship to the Energy and Nitrogen Balances of the Ewes fed on Diet 541*

During the 12 months of the observations each of the ewes traversed the physiological states that were mentioned in the introduction to this paper; their nutritional status ranged from one of frankly negative energy balance in which

TABLE 1
THE RATE OF WOOL GROWTH AT DIFFERENT NUTRITIONAL LEVELS

Sheep No. and Surface Area	Period	Dry Clean Wool Fibre			
		W (obs.) g.	Weight g./day	D, Mean Diameter μ	Relative Length
		(1)	(2)	(3)	(4)
522 (0.87 m. ²)	1	0.50	1.5	13.85 \pm 0.124	100
	2	0.49	1.4	14.75 \pm 0.136	86
	3	1.55	4.6	18.79 \pm 0.159	168
	4	2.35	7.0	21.60 \pm 0.175	193
547 (0.85 m. ²)	1	0.46	1.3	14.84 \pm 0.060	100
	2	0.60	1.7	16.35 \pm 0.073	107
	3	1.22	3.5	18.84 \pm 0.076	165
	4	2.48	7.2	21.80 \pm 0.080	250
558 (0.84 m. ²)	1	0.40	1.1	13.18 \pm 0.105	100
	2	0.50	1.4	15.15 \pm 0.125	95
	3	1.68	4.8	20.62 \pm 0.147	172
	4	2.65	7.6	22.27 \pm 0.136	232
550 (1.05 m. ²)	1	0.27	1.0	13.13 \pm 0.092	100
	2	0.57	2.0	15.81 \pm 0.106	145
	3	0.91	3.2	17.80 \pm 0.116	183
	4	1.34	4.8	20.17 \pm 0.122	210
559 (1.05 m. ²)	1	0.45	1.6	15.73 \pm 0.128	100
	2	0.48	1.7	16.27 \pm 0.129	99
	3	1.24	4.4	19.56 \pm 0.149	178
	4	1.83	6.5	22.00 \pm 0.139	208
572 (1.05 m. ²)	1	0.43	1.5	15.40 \pm 0.143	100
	2	0.48	1.7	15.95 \pm 0.165	104
	3	1.98	7.1	20.53 \pm 0.189	260
	4	2.45	8.7	21.42 \pm 0.202	295
560 (1.00 m. ²)	1	0.48	1.6	16.08 \pm 0.134	100
	2	0.75	2.5	18.05 \pm 0.138	124
	3	1.18	4.0	18.69 \pm 0.154	182
	4	1.58	5.4	20.83 \pm 0.148	196

(1) = observed weight of clean dry wool produced on 150 cm.²/14 days.

(2) = weight of clean dry wool produced/day, computed from (1), *vide* text.

(3) = mean diameter of fibres on patch.

(4) = relative mean length of fibres computed from W/D^2 , period 1 being taken as 100.

they were drawing on their own tissues to the extent of approximately 500 kg. cal./day, through two intermediate levels, to one of strongly positive energy balance which favoured the rapid production of tissue protein and fat. The supply of protein from the fodder was increased approximately 400 per cent.

from the lowest to the highest level of feeding (Table 2), but coincident with this increase the overall nutritional status was altered in favour of other physiological processes which competed seriously with the follicles for the supply of substrate. The rate of wool production under these conditions was clearly the resultant of a number of variables, the nature of which changed with the level of feeding – the substrate of amino acids was depleted at the lower levels to provide fuel to meet the energy demands, and at the higher levels, to provide for the competing

TABLE 2
NITROGEN BALANCE AND WOOL GROWTH OF SHEEP ON DIET 541

Sheep No.	Period	Intake (g.N/24 hr.) N_i	Faeces (g.N/24 hr.) N_f	Urine (g.N/24 hr.) N_u	Available N (g.N/24 hr.) N_a	N Balance (g.N/24 hr.) N_b	Wool (g.N/24 hr.) N_w	Energy Balance (kg.cal./24 hr.)
522	1	3.67	0.69	5.22	5.47	-2.24	0.25	-405
	2	8.08	1.44	5.88	6.64	+0.76	0.24	-20
	3	12.90	3.06	7.32	9.84	+2.52	0.77	+250
	4	19.83	4.19	10.00	15.64	+5.64	1.17	+780
547	1	3.48	0.64	5.45	5.67	-2.61	0.22	-430
	2	7.86	1.48	5.71	6.38	+0.67	0.29	-35
	3	12.51	2.53	7.44	9.98	+2.54	0.59	+380
	4	19.26	3.81	9.77	15.45	+5.68	1.20	+715
558	1	3.38	0.60	5.00	5.19	-2.22	0.19	-395
	2	7.97	1.90	5.80	6.07	+0.27	0.24	-70
	3	12.60	2.78	7.22	9.82	+2.60	0.81	+230
	4	18.20	4.30	9.38	13.90	+4.52	1.28	+715
550	1	4.18	0.63	5.19	5.35	-1.64	0.16	-480
	2	9.55	1.65	7.50	7.90	+0.40	0.34	-30
	3	15.05	2.60	10.45	12.45	+2.00	0.54	+280
	4	18.85	4.10	12.10	14.75	+2.65	0.80	+640
559	1	3.95	0.72	4.79	5.06	-1.56	0.27	-500
	2	9.42	1.65	7.80	7.77	-0.03	0.29	-100
	3	15.55	3.14	10.35	12.41	+2.06	0.74	+230
	4	20.30	5.52	11.45	14.78	+3.33	1.10	+730
572	1	4.62	0.73	6.10	6.36	-2.21	0.26	-430
	2	9.42	2.02	7.15	7.40	+0.25	0.29	-30
	3	15.20	2.81	10.25	12.39	+2.14	1.19	+270
	4	19.80	5.38	11.30	14.42	+3.12	1.47	+560

In period 1 of each series when the animals were frankly in negative balance the available nitrogen, N_a , is expressed as the nitrogen in the urine, N_u , plus the nitrogen in the wool, N_w , which under these circumstances is the nitrogen turnover. In all other periods N_a is the nitrogen intake, N_i , minus the nitrogen in the faeces, N_f . The nitrogen balance, N_b , in each case is $N_i - (N_f + N_u)$. The nitrogen in the wool, N_w , is computed (*vide text*) from the wool collected by shaving 150 cm.² patches outlined by tattoo lines on either shoulder, the nitrogen content of pure dry wool keratin being taken as 16.8 per cent. The energy balance figures are those reported by Marston (1948). The levels of feeding were changed in ascending steps for Nos. 522, 547, and 558, and in descending steps for Nos. 550, 559, and 572.

syntheses involved in the laying down of tissue protein. Thus the relationships between the rates of wool production and the intakes of protein (or, more precisely, of that part of the ingested protein which became available to the animals), although roughly linear (*vide* Fig. 3.) were not simple ones.

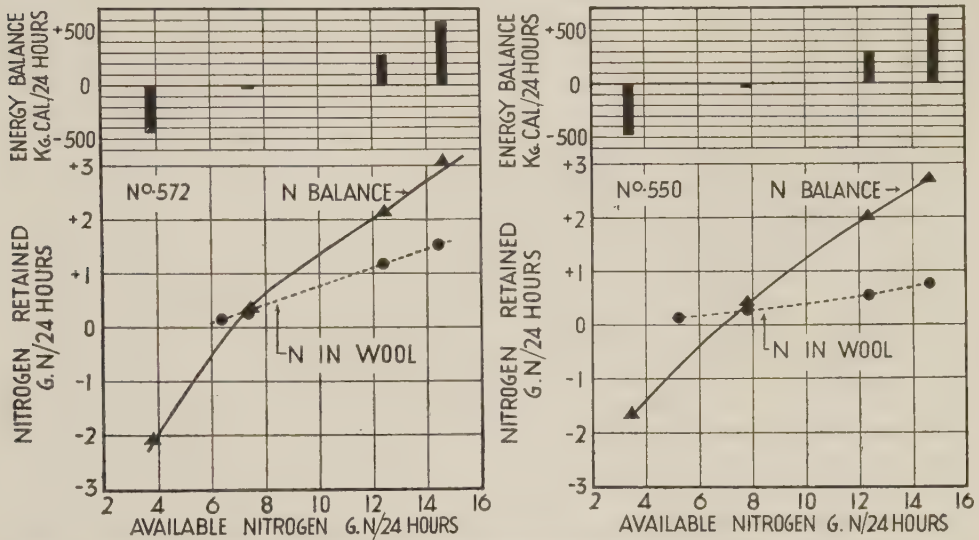


Fig. 3.—The difference in the wool producing efficiency of ewes No. 572 and No. 550 over a range of practically identical nutritional conditions is shown. Neither ewe expressed its full capacity as a wool producer under the conditions imposed. Above the maintenance level of fodder intake the relative rates of the synthesis of wool keratin and muscle protein are indicated respectively by the nitrogen in the wool and the difference between this and the total nitrogen retained. For animals in frankly negative balance, the nitrogen available for wool production was computed (*vide* footnote to Table 2) from the nitrogen in the urine plus the nitrogen in the wool. Under such conditions this is a close estimate of the nitrogen turnover. The amino acids arising from the tissue proteins which are degraded when the animal is in negative energy balance add to the substrate available for wool growth — when a sheep is living entirely at the expense of its own tissues, after prolonged fasting, its wool continues to grow at a considerable rate — and so at fodder intake levels below maintenance (those of period 1) the rate of wool growth bears a closer relationship to the nitrogen turnover estimated in this way than to the nitrogen available from the fodder. In the curves which refer to the state of nitrogen balance of the animals, however, the “available nitrogen” at the intake level of period 1, as at all other levels, is computed from the difference between the nitrogen in the fodder and the nitrogen in the faeces. This is a reasonably close estimate of the nitrogen in that part of the fodder that is digested and absorbed. The relatively small contribution made to the faecal nitrogen by nitrogenous constituents excreted into the bowel and not reabsorbed has been neglected in these considerations.

At high levels of protein intake the increment in the rate of wool growth might be expected to diminish towards an asymptote which would be defined by the maximum capacity of the follicles to utilize amino acids.* There was no

* Although this maximum is primarily a hereditary characteristic of the individual it may be influenced materially by early nutritional history, for obviously a sheep that has been stunted through malnutrition during its period of rapid growth would never express its full hereditary propensity as a wool producer.

evidence, however, that the maximum rate of wool production was approached in the range of nutritional conditions to which the experimental animals were subjected: the relationship between wool production and protein intake remained, in every case, approximately linear at intake levels above maintenance. It may be concluded that under these conditions the rate of wool growth was limited not by the maximum ability of the follicles to utilize amino acids but by the supply of raw materials for the synthesis of keratin.

(d) *The Efficiency of the Merino as a Converter of Protein to Wool Fleece*

At the highest level of feeding when the protein intake was comparatively large and the state of the energy balance was such as to minimize the immediate call on the amino acids for fuel, the nitrogen retained in the wool was less than 10 per cent. of the nitrogen absorbed from the ration — it varied between 10 per cent. of the available nitrogen (No. 572) and 5.4 per cent. (No. 550). Thus it is evident that the capacity of the ingested protein to provide the essential amino acids for the synthesis of wool keratin was seriously limited. Although at the higher levels of food intake a considerable drain on the substrate of amino acids was imposed by the syntheses involved in flesh formation, this complication did not materially alter the situation. The extent of the depletion may be determined with reasonable accuracy from the difference between the total nitrogen retention and the nitrogen content of the wool produced (*vide* Table 2); if the nitrogen available to the follicles is estimated in this way, the efficiency of its conversion to wool was at its highest, 12 per cent., in No. 558 which was laying down flesh during period 4 more rapidly than any of the other animals, and at its lowest, 6.2 per cent., in No. 550 which throughout was the poorest wool producer.

The capacity of diet 541 to provide essential amino acids for wool and flesh production respectively may be estimated from the composition of the main proteins concerned in these transactions (Table 3). It is apparent that the sulphur-containing amino acids, cystine and methionine, would impose the first limiting factor for the synthesis of keratin from the assemblage of amino acids available from diet 541. The overall composition of this substrate would be influenced by differences in the digestibility of the proteins from the wheat and the lucerne, but the error involved in assessing, from the composition of the diet and the nitrogen absorbed, its ability to provide the essential amino acids for wool production would be negligible as the proteins from lucerne and wheat have practically an identical capacity to provide cystine.*

If the potential cystine were transferred quantitatively to wool keratin the overall efficiency of conversion of the amino acids from diet 541 could not exceed 27 per cent. The proportions converted were much lower than this. The greatest observed efficiency was achieved by No. 558. At the highest level of feeding this

* It is within the scope of the animal organism to produce cystine from methionine through the intermediary thio-ether, $\text{H-S-(}\beta\text{-amino-}\beta\text{-carboxyethyl)-homocysteine}$: the serine involved in this conversion being formed *in vivo* either from other amino acids or by transamination. The potential capacity of an amino acid mixture to supply cystine to the animal is thus the sum of its contents of methionine and cystine, 1 mol. of methionine being capable of transformation to 1 mol. (80 per cent. of its weight) of cysteine. The reaction is not reversible (for review of literature see Marston 1946, p. 207, *et seq.*).

TABLE 3

THE DISTRIBUTION OF AMINO ACIDS IN THE PROTEIN SOURCES OF DIET 541 AND THE PRODUCTS OF THE NUTRITIONAL TRANSACTIONS

Amino Acid	Per cent. of Amino Acid Nitrogen in Total Nitrogen of				
	Whole Wheat Protein (1)	Lucerne Protein (2)	Diet 541 Protein (3)*	Wool Protein (4)	Whole Muscle Protein (5)
Cystine	1.3(b)	1.2(d)	1.2	9.0(e)	0.8(a)
†Methionine	1.5(b)	1.3(d)	1.4	0.4(f)	1.9(a)
†Threonine	2.4(b)	4.0(b)	3.4	4.7(f)	3.9(a)
Arginine	8.4(b)	15.1(d)	12.5	20.0(e)	15.3(a)
†Histidine	3.6(b)	2.3(d)	2.8	1.1(e)	4.1(a)
†Lysine	3.2(b)	6.6(d)	5.2	3.2(e)	10.4(a)
†Tryptophan	1.0(b)	1.8(d)	1.5	0.6(e)	1.0(b)
†Phenylalanine	3.0(b)	2.4(b)	2.6	1.9(f)	2.6(b)
Tyrosine	2.3(b)	2.5(d)	2.4	2.0(e)	1.5(b)
†Leucine	4.5(b)	7.4(b)	6.2	†7.2(f)	5.3(b)
Isoleucine	2.4(b)	3.6(b)	3.1		4.2(b)
†Valine	3.4(b)	4.5(b)	4.1	3.4(f)	4.3(b)

* Computed from (1) and (2) on basis 100 g. of diet 541 contained 2.3 g. protein N of which 0.92 g. N (40 per cent.) was supplied by wheat and 1.4g. N (60 per cent.) by lucerne.

† Essential amino acids.

‡ The sum of leucine and its isomer.

References

- (a) Beach, E. F., Munks, B., and Robinson, A. (1943).—*J. Biol. Chem.* **148**: 431.
- (b) Block, R. J., and Bolling, D. (1947).—"The Amino Acid Composition of Proteins and Foods." (Thomas: Ill., U.S.A.)
- (c) Block, R. J. (1939).—*J. Biol. Chem.* **128**: 181.
- (d) Lugg, J. W. H. (1946).—*Aust. Chem. Inst. J.* **13**: 88.
- (e) Marston, H. R. (1928).—*Coun. Sci. Industr. Res. Aust. Bull.* No. 38.
- (f) Martin, A. J. P., and Syngé, R. L. (1941).—*Biochem. J.* **35**: 91, 294.

animal bound in its wool 47 per cent. of the potential cystine available in the substrate that remained after requirements for flesh formation** had been subtracted from the total amino acids absorbed. The efficiency *decreased* materially at lower levels of food intake. In period 4, the highest level, it ranged between 47 per cent. and 24 per cent.; in period 3 it ranged between 40 per cent. and 19 per cent.; and at the approximate maintenance of period 2 it ranged between 18 per cent. and 14 per cent. The low efficiency of conversion at maintenance level is simply explained by the relatively great call on the amino acids to serve as fuel to support the basal energy requirements. This call was not inconsiderable at the intermediate level of feeding, period 3, for under these conditions the combustible energy of the absorbed amino acids either approximated closely or actually exceeded the calorific value of the retained nutrients.

As the follicles have little or no capacity to store their requirements of amino acids, wool growth is essentially dependent on the concentration of amino acids in the tissue fluids. The continual high concentration of amino acids in the

** Assessed, *vide supra*, from the difference between the total nitrogen balance and the nitrogen in the wool.

systemic blood stream which apparently is necessary to sustain maximum wool growth tends to favour wastage of the substrate by oxidative deamination, even at food intakes which ensure a strongly positive energy balance. This tendency is enhanced by the considerable diurnal variations in the rate of absorption of amino acids from the intestine. The relative rates of absorption of amino acids and of their utilization by the follicles probably comprise the main factor responsible for the low efficiency of utilization of the sulphur-containing amino acids observed at the highest levels of feeding in these experiments.

(e) The Influence of Fodder Intake on the Dimensions of the Wool Fibre

As there was no evidence that any of the follicles ceased to function during the period of observation — there was no sign of cast fibres in the staple even at the lowest level of production — it may be assumed that the density of the fibre population on the area from which the wool was collected remained the same at all levels of feeding and so the relative change in the mean length of the fibres at any level of feeding may be derived, provided the specific gravity of the fibres remains unaltered, from the expression, $L = W/kD^2$, where L = the mean length, D = the mean diameter, and W = the weight produced in the observed period, k being a constant. The data relevant to the influence of fodder intake on the dimensions of the fibre are set out in Table 1.

It is obvious that the rate of wool production is contingent on a change in the mean diameter of the fibres: as $W = kD^2L$, W being related to D by a function considerably in excess of D^2 , an alteration in mean diameter of the fibres reflects a relatively large change in the rate of wool production.

The variance of the fibre diameter population was also subject to the level of food intake — “g” statistics revealed that the fibre diameter distributions, which in every case tended to be skewed towards the stronger classes became, as the rate of wool growth increased, more asymmetrical and more platykurtic. The findings suggest that the finer classes change over a more restricted range than the stronger classes; apparently, with an alteration in the rate of growth, each fibre is influenced up to the limit of its capacity in proportion to the area of its cross section. Detailed analyses of these variations will be discussed in another paper.

(f) Wool Production by Grazing Sheep

From the above considerations of the physiological factors involved in wool production, it would appear unlikely that a high-producing Merino sheep could ever express its full propensity for wool production while grazing on natural pastures.

Few pastures, at any stage of their growth, would be capable of providing the nutritional requirements for maximum wool production. When their protein content is greatest the fodder plants are young and succulent and the sward is usually wet; in these circumstances the quantity of the available fodder with which a sheep can deal is limited by the water in (and on*) the pastures. When

* To derive its energy requirements from constituents other than the available protein of wet young pastures a sheep would, of necessity, ingest between 6 and 10 litres of water each day.

they are dry, their protein content is low and less easily assimilated from the lignified plant cells. The bulk of this relatively indigestible fodder that a sheep would need to consume in order to provide the full complement of amino acids for maximum wool production, exceeds the capacity of its digestive organs. Under these extremes of grazing conditions the situation is complicated further by the inability of the sheep to fulfil its basal energy requirement even when it has fed to repletion. The supply of amino acids which otherwise would provide a substrate for wool production is then depleted to serve as fuel.

The ability of a pasture to provide the nutritional requirements for maximum wool growth, if it were ever capable of achieving this, would be transitory; and so it may be concluded that the wool production of a grazing Merino is usually, if not always, limited by the nutritional quality of its fodder.

Considerable changes in the rate of wool production might thus be expected to supervene on the seasonal fluctuations in the chemical composition of the pastures. These changes would be reflected in the mean diameter of the wool fibres.

This phenomenon, which is prevalent under natural grazing conditions in Australia, is evident from the observations set out in Figure 4. The mean fibre diameters of the wool grown on areas delineated by tattoo lines on the shoulders of a group of ten high-producing Merino ewes were determined* at monthly intervals for two years. The animals were of the same breeding as those employed in the experiments described above. They were members of the Division's breeding flock and grazed with it on the natural pastures of the Adelaide foothills. The performances of the three reported in Figure 4 are representative of the range of variations observed. The very considerable seasonal change in the fibre diameters of all is obvious, and although no quantitative measure of the chemical composition of the pastures was attempted, it is clear that the changes in the fibre diameters reflected very closely the nutritive quality of the fodder of which there was always a large excess available to the animals.

As the experimental observations discussed above have proven that the rate of wool growth in this type of Merino is proportional to a function considerably greater than the square of the mean fibre diameter, the relative changes in the rates at which wool was produced at different times of the year by the grazing flock were obviously large. By comparison with the performance of the individuals fed under laboratory conditions, it would appear that the nutritional state of the grazing ewes varied with the season over a range which at its lowest was somewhat better than that of period 2, and at its highest materially superior to period 4 (*vide supra*), the former being reached in midwinter and the latter in late spring and early summer. The actual nutritive quality of the fodder if judged from the chemical composition of the dried pasture clips would have been greatest in midwinter when the fodder plants were in their earlier stages

* The mean diameter was derived by measuring at random the dried clean fibres (*vide supra*) from a sample collected by clipping the 15 cm. x 10 cm. area with very fine hair-clippers, the patch having been clipped similarly a week previously. Each reported mean and its variance thus refers to approximately one week's growth at the time indicated. Five hundred fibres were measured in each case.

of growth. The pastures then, however, were usually sodden with moisture and so the *quantity of dry matter consumed by the grazing animal*, and not the quality of the fodder, became the first limiting factor. The steady decline in the

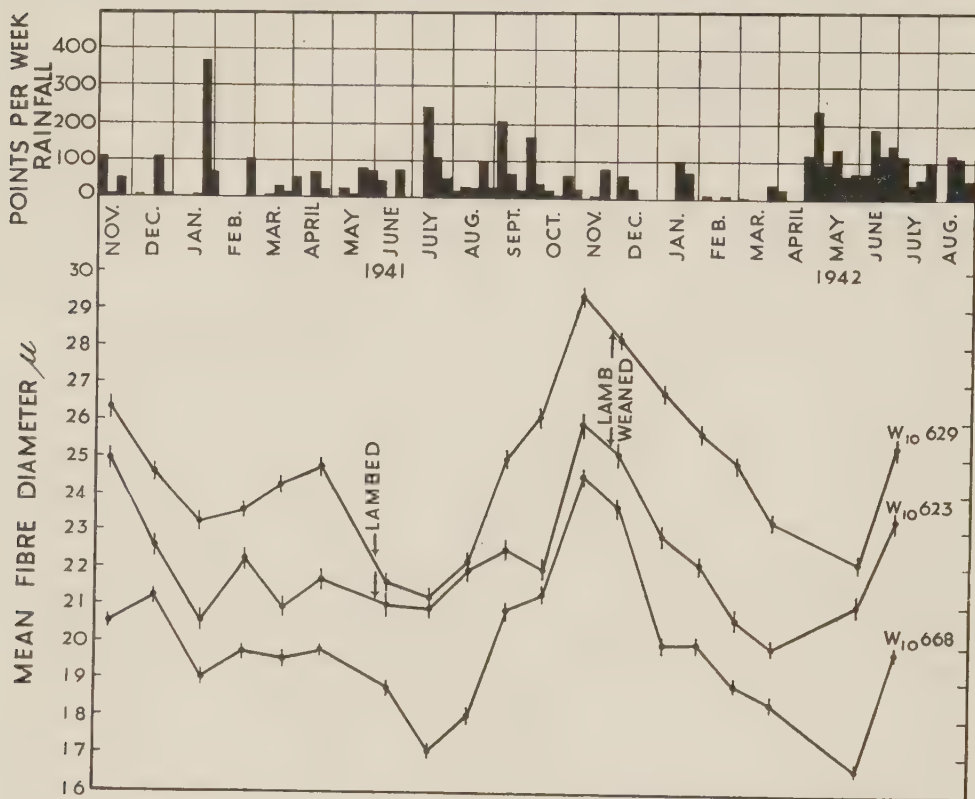


Fig. 4.—The seasonal variation in wool production by sheep grazing on natural pastures composed essentially of annual species is shown. The mean fibre diameters of the wool grown on areas delineated on the shoulders of each animal are plotted as points with \pm P.E. indicated by the length of the line on either side. The change in mean fibre diameter between the winter level (June-August) and that of the flush of spring (Oct.-Nov.) implies approximately a fourfold increase in rate of wool production.

Ewes No. 629 and No. 623 carried lambs. Ewe No. 668 did not lamb in 1941. A supplement of barley (4 oz. per day) was available to all ewes from January 21 to July 3, 1941, and from January 5 to July 7, 1942.

rate of wool growth during the summer reflected the decreasing nutritional quality of the available fodder. The sheep is a selective feeder and so would deplete the dry, standing pasture of its most nutritive constituents.

IV. GENERAL CONCLUSIONS

The efficiency with which a high-producing Merino sheep converts its fodder protein to wool fleece obviously is influenced by a number of physiological factors, and while there can be no doubt that the initial nutritional limitation is imposed by the potential capacity of the fodder to provide cystine, the ultimate supply of essential amino acids that becomes available to the follicles

is clearly subject to competitive reactions which change both in nature and extent with the overall nutritional status of the animal.

It may be concluded from the above experimental observations that wool production is practically always limited by nutritional factors. Both the quantity and quality of the fodder consumed by a grazing sheep vary over a wide range according to the season, and these variations are reflected in the fleece by alterations both in the length and in the mean diameter of the wool fibres. The response of individual follicles is by no means constant, and so alteration of the nutritional level of the sheep leads to a marked change in the nature of the fibre diameter distribution curve. Thus, attempts to classify a sheep by the mean and variance of the fibre diameters of its wool fleece are not convincing. It would not be justifiable from the elaborate data of these experiments either to assess precisely the wool-producing propensity of any of the individuals or to predict the way in which these sheep would react to a nutritional environment distinct from the observed range.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

- MARSTON, H. R. (1935).—*J. Agric. Sci.* **25**: 103-12.
MARSTON, H. R. (1946).—"Nutrition and Wool Production." Symposium on Fibrous Proteins, published by the Society of Dyers and Colourists, Leeds, p. 207 *et seq.*
MARSTON, H. R. (1948).—*Aust. J. Sci. Res. B* **1**: 93-129.

NUTRITIONAL FACTORS INVOLVED IN WOOL PRODUCTION BY MERINO SHEEP

II. THE INFLUENCE OF COPPER DEFICIENCY ON THE RATE OF WOOL GROWTH AND ON THE NATURE OF THE FLEECE

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(Plate 1)

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Summary

A range of nutritional states which varied in relatively small degrees from a normal physiological condition to one of acute copper deficiency was induced in a series of evenly matched groups of Merino sheep depastured on deficient terrain by providing them with supplements of copper which extended through sub-optimum amounts to quantities in excess of their full requirements.

The copper and molybdenum intakes from the pastures were assessed and possible interactions are discussed.

The rate at which the syndrome of copper deficiency developed was governed by the quantity of copper provided in the supplement.

The amount of wool produced by the unsupplemented animals was materially less than that produced by those which received the copper supplements, the increments of increase in wool production above that of the unsupplemented level being related to the additional copper in the supplements by a curve of diminishing returns which reached the asymptote of wool production when the equivalent of between 7.5 and 10 mg. Cu/day was provided.

The capacity to impart crimp to the fibres began to be influenced when the concentration of copper in the systemic blood stream fell below approximately 0.4 mg. Cu/l. and this function failed completely at blood-copper levels <0.2 mg./l. A normal concentration of copper in the bloods of all animals which received supplements ≥ 10 mg. Cu/day was maintained and no untoward effects were suffered by these animals.

I. INTRODUCTION

Previous experimental observations (Marston 1946; Marston and Lee 1948; Marston, Lee, and McDonald 1948*a*, 1948*b*) have revealed that the first discernible symptom of an abnormally low copper status in the sheep is the failure of the follicles to impart crimp to the wool fibres.

As these earlier experiments were designed primarily to study symptoms other than the impairment of wool growth, the groups of experimental animals were made up solely on the basis of body weight, and no special precautions were taken either to select them from a flock of particularly even wool type or to distribute the individuals among the groups according to their wool producing capacities. Although the lesions always appeared in the fleeces of the copper-

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deficient animals the individual variation in the fleece weights within the groups was large, and so discussion of the effects of copper deficiency on the overall wool producing capacity of the sheep was withheld until more conclusive data were available.

In the experiment described below, the approach was analogous to a chemical titration. Supplements of copper ranging in increments from nil to an amount that previous experiments (*ibid.*) had indicated would more than fulfil the physiological requirements of sheep depastured on the deficient terrain where the experiment was conducted, were administered to a series of evenly matched groups of sheep. In this way a graded series of deficiency states was realized. The "end point" after which there was no further response to additional copper was indicated quantitatively by the mean weight of clean scoured wool produced and qualitatively by the complete absence of copper-deficient lesions in the fleeces of the individuals of the particular group.

II. THE EXPERIMENTAL ANIMALS

The experimental animals were chosen from a flock of over 5,000 evenly bred (Mutooroo) Merino ewe hoggets by drafting off 140 of desirable size and conformation, and then by individual examination selecting 70 of them for similarity of fleece characteristics. These were tagged and transported by rail to the field station where they were received on to the deficient pastures early in August 1944. A fortnight later a staple of wool was taken from the skin region above the acromion of the right scapula of each individual and the mean fibre diameter of the base of this staple was determined by direct measurement. The animals were ranged *seriatim* according to the fibre diameters of their fleeces and, after rejection of the extremes, the remaining 64 were bracketed consecutively into 8 lots each of 8 animals. The individuals of each lot were then distributed at random (Tippett) through the 8 experimental groups which thus were as evenly matched as was feasible. The mean age of the animals when they were transferred to the deficient pastures was approximately 10 months; they were about one year old when treatments began. The observations were continued for 4 years; the behaviour of the animals during the first 2 years is reported in this paper.

III. THE PROCEDURE

The procedure adopted was similar to that described in previous papers. The 8 groups were depastured as one flock on the deficient terrain at Robe where it had been demonstrated that sheep suffer a dual deficiency of cobalt and of copper and that appropriate treatment with either element would reveal the uncomplicated syndrome of a profound deficiency of the other. Cobalt deficiency was prevented by providing all animals with an ample supplement of cobalt. The amounts of copper administered to the groups were chosen to range in relatively small increments about the quantity previously proven adequate to provide indefinitely for all physiological functions of sheep depastured on this terrain. The copper and cobalt were administered as 5 ml. of an aqueous solution

of cupric sulphate and cobaltous sulphate in concentrations such as to supply amounts equivalent to those set out below:

Group	Treatment
1-Mu-0	1 mg. Co/day
2-Mu-1	1 mg. Co/day + 1 mg. Cu/day
3-Mu-2.5	1 mg. Co/day + 2.5 mg. Cu/day
4-Mu-5	1 mg. Co/day + 5 mg. Cu/day
5-Mu-7.5	1 mg. Co/day + 7.5 mg. Cu/day
6-Mu-10	1 mg. Co/day + 10 mg. Cu/day
7-Mu-15	1 mg. Co/day + 15 mg. Cu/day
8-Mu-20	1 mg. Co/day + 20 mg. Cu/day

The flock was mustered thrice weekly (Sun., Wed., Fri.) and the doses administered *per os* by means of a syringe in a manner which ensured that the whole of the solution would be swallowed. At monthly intervals the concentrations of copper and of haemoglobin in the blood of each individual were determined in 20 ml. samples withdrawn with special precautions from the jugular. Pasture clips were collected at these intervals from the paddocks on which the animals had grazed and from composite samples the concentrations of copper and of other trace elements were determined.

IV. THE EXPERIMENTAL FINDINGS

(a) *The Copper and Molybdenum Content of the Pastures*

The concentration of copper in the composite pasture samples fluctuated erratically about a mean of approximately 3 μ g. Cu/g. dry wt. (Fig. 1). This vacillation, however, was the consequence of changes in botanical composition of the pastures rather than of transient alterations in the chemical composition of individual plants—the sheep were grazed in rotation over several paddocks and the samples were taken from the pastures on which they were feeding at the time when the blood samples were collected.

The fact that plant species differ widely in their ability to assimilate minor elements from the soils on which the experimental animals were grazed became obvious early in these investigations. The actual and relative amounts of the trace elements (Co, Cu, Fe, Zn, Mo, etc.) that are concentrated within the tissues of a plant were found to be influenced by three main factors—the species, the stage of development of the plant, and the prevailing nutritional environment of the soil in which it is grown.

Although the pastures on the dunes where the experimental flocks were confined consist essentially of a very simple association of the indigenous grasses, *Lagurus ovatus* and *Bromus madritensis*, a sparse growth of herbaceous plants appears during the winter and spring, and some of these undoubtedly are consumed by the grazing sheep. This herbage contained considerably more copper than the grasses in which the concentration varied only within the rather restricted range of from 2 μ g. to 3 μ g. Cu/g. dry wt. While it is not possible to predict with confidence either the amount of copper in the fodder selected by the sheep from pastures of mixed composition or to assess the extent to which copper retention is complicated by the presence of other elements in this fodder, it may be assumed

that for most of the year by far the greater bulk of the pasture ingested by the experimental animals consisted of grasses, and so the average intake of copper was between 2.5 and 4 mg. Cu/day, i.e. between one-third and one-half of the amount of copper ingested by sheep of similar weight from pastures on areas where copper deficiency symptoms are never experienced.

There is evidence that the retention of copper by the ruminant is influenced by the ingestion of molybdenum — the malady, suffered by cattle on "teart" pastures, which has been claimed to result from chronic ingestion of relatively large quantities of molybdenum, responds to therapy with copper (Ferguson, Lewis, and Watson 1940, 1943), and Dick and Bull (1945) have demonstrated unequivocally that the copper reserves in the liver of a sheep may be depleted by adding ammonium molybdate to the fodder. The quantity of molybdenum involved in "teart" pastures, however, greatly exceeds that present in the pastures on which the following experiments were conducted.

In the alkaline *milieu* of the calcareous littoral, the enhanced availability of molybdenum tends strongly to favour its concentration within the tissues of certain species of plants, and there was certainly more molybdenum in the fodder plants from the dunes on which the experimental flocks were grazed than in similar plants grown elsewhere on more acid soils. Its concentration in the indigenous grasses which grew there varied, according to the season, between the approximate limits of 1.2 and 4.0 $\mu\text{g. Mo/g. dry wt.}^*$ It became concentrated to a greater extent in the herbaceous plants and for a period during winter and spring the composite pasture samples contained approximately 8 $\mu\text{g. Mo/g. dry wt.}$ The molecular ratios of Mo/Cu in these samples rarely exceeded unity, but in the spring of both 1945 and 1946 this ratio increased to approximately 1.6.

At this juncture the influence exerted by these quantities of molybdenum on the capacity of the sheep to retain copper is not clear. But, whether the reduction of the copper status of the experimental sheep depastured on these areas was the result of a dietary deficiency *per se*, or whether it was in part imposed by the interaction of molybdenum or of other constituents of the fodder, there can be no doubt that the *syndrome which developed was the result of copper deficiency*.

(b) Influence of Copper Deficiency on the Body Weight

It was not feasible to draw definite conclusions from the relatively small differences which became evident between the mean body weights of the groups that received copper supplements. As the groups had been matched originally according to their wool characteristics, the mean body weights at the beginning of the treatments varied over a wider range than in previous experiments, and from their subsequent behaviour it was apparent that in so far as the propensity for further growth was concerned some groups had inadvertently been biased by this method of selection. However, with the exception of the aberrant behaviour of two groups (4-Mu-5 and 6-Mu-10), the mean body weights after two years of treatment ranged in order of the amounts of copper provided in the supplements.

* Molybdenum was determined by the method of Dick and Bingley (1947).

Three months after the beginning of the treatments, the mean body weight of the group which received no additional copper was significantly lower than that of any of the copper-supplemented groups, and subsequently this difference became progressively greater (Fig. 1).

(c) *The Influence of Copper Deficiency on the Copper Content of the Blood*

The animals had been depastured on the deficient terrain for approximately 3 months before treatments began, and during this period the concentration of copper in their bloods had fallen to a relatively low level. They had received their first supplementary drenches the day before the first blood samples were taken. The relatively rapid reinstatement of a normal blood-copper level in the groups which were given an adequate supply of copper and the further steady fall in the level in those which received less copper than would provide the full requirements of sheep on this terrain are obvious from Figure 1. The blood-copper levels of all groups fluctuated according to the seasonal changes in the composition of the available fodder but never sufficiently to confuse the order in which they reflected the concentrations of copper provided in the supplements.

Analysis of variance of the concentrations of copper in the bloods of all groups (Table 1) reveals clearly the fact that the supplements containing less

TABLE 1
EFFECT OF COPPER SUPPLEMENTS ON THE COPPER CONCENTRATION OF THE BLOOD

Date of Collection	Mean mg. Cu/l. Blood							
	Treatment, mg. Cu/day							
	0	1.0	2.5	5.0	7.5	10.0	15.0	20.0
18.xi.44	0.29	0.40	0.45	0.39	0.29	0.51	0.34	0.43
19.xii.44	0.32	0.45	0.40	0.61	0.65	0.79	0.79	0.58
15.i.45	0.26	0.39	0.43	0.51	0.70	0.59	0.65	0.69
20.ii.45	0.23	0.31	0.38	0.48	0.51	0.51	0.62	0.63
20.iii.45	0.23	0.33	0.47	0.62	0.67	0.81	0.98	0.82
17.iv.45	0.16	0.27	0.43	0.55	0.50	0.73	0.78	0.70
15.v.45	0.24	0.36	0.49	0.62	0.72	0.81	0.83	0.82
19.vi.45	0.16	0.26	0.39	0.51	0.65	0.80	0.89	0.82
17.vii.45	0.16	0.22	0.32	0.36	0.63	0.73	0.87	0.80
21.viii.45	0.14	0.15	0.28	0.34	0.48	0.62	0.74	0.68
18.ix.45	0.11	0.14	0.17	0.29	0.37	0.69	0.82	0.81
24.x.45	0.12	0.15	0.21	0.38	0.57	0.89	1.12	1.13
22.xi.45	0.08	0.09	0.15	0.23	0.45	0.57	0.62	0.60
20.xii.45	0.15	0.18	0.24	0.52	0.75	0.81	0.89	0.83
22.i.46	0.16	0.22	0.33	0.53	0.67	0.80	0.73	0.80
21.ii.46	0.13	0.16	0.32	0.50	0.73	0.73	0.72	0.77
24.iii.46	0.11	0.13	0.18	0.33	0.46	0.55	0.63	0.72
25.iv.46	0.12	0.13	0.17	0.32	0.45	0.57	0.82	0.77
11.vi.46	0.12	0.15	0.19	0.22	0.44	0.67	0.83	0.93
28.vii.46	0.14	0.11	0.13	0.16	0.45	0.68	0.84	0.72
3.ix.46	0.08	0.20	0.10	0.21	0.41	0.63	0.74	0.79
19.ix.46	0.14	0.25	0.16	0.32	0.49	0.77	0.86	0.99

Significant differences between means within the table:	Probability		
	0.05	0.01	0.001
	0.12	0.16	0.20 mg. Cu/l.

than the optimum concentration of copper merely delayed the depletion of the copper reserves of the animals and that after approximately 2 years had elapsed the blood-copper levels, even of those groups which received supplements which approximated the quantity necessary to fulfil their requirements, fell steadily and approached those of the unsupplemented group. At the end of the first year there was no significant difference ($P < 0.01$) in this respect between the unsupplemented animals and those of the groups that received an additional 1 mg. and 2.5 mg. Cu/day. After 2 years, the mean blood-copper concentrations of the group that received 5 mg. Cu/day had fallen to a level which was not significantly different from those of the three groups which received less copper. The animals that received the equivalent of 10 mg. Cu/day showed no tendency to become depleted in this way.

(d) *The Influence of Copper Deficiency on the Haemoglobin Content of the Blood*

During the first year of the experiment no significant degree of anaemia developed in the animals of group 1-Mu-0 although over practically the whole of this time the blood-copper level was below 0.2 mg. Cu/l. In the flush of the spring of 1945, however, the individuals of this group became anaemic. The

TABLE 2

THE EFFECT OF COPPER SUPPLEMENTS ON THE OXYGEN CARRYING CAPACITY OF THE BLOOD

Date of Collection	Mean. vol. O ₂ /100 ml. Blood							
	Treatment, mg. Cu/day							
	0	1.0	2.5	5.0	7.5	10.0	15.0	20.0
18.xi.44	12.51	12.73	12.21	12.24	12.00	11.70	12.16	11.95
19.xii.44	12.35	12.65	12.64	12.60	12.68	12.16	13.21	12.68
15.i.45	12.85	12.94	13.36	12.23	13.49	12.46	13.53	13.23
20.ii.45	13.11	13.01	12.66	12.50	13.34	12.45	13.83	13.68
20.iii.45	11.56	12.29	12.48	12.35	12.69	11.71	12.85	12.83
17.iv.45	11.56	12.86	12.43	11.81	12.70	11.74	12.46	12.39
15.v.45	11.80	12.63	12.34	11.71	13.30	12.26	12.78	12.68
19.vi.45	11.10	11.93	12.59	11.86	13.30	12.29	13.35	13.06
17.vii.45	11.18	11.70	12.15	11.63	12.58	11.88	13.11	13.04
21.viii.45	10.51	10.85	11.96	11.43	12.00	11.41	12.46	11.88
18.ix.45	10.20	11.29	12.15	11.84	12.78	12.00	12.96	12.75
24.x.45	7.36	10.34	10.11	12.17	12.39	12.49	13.55	12.95
22.xi.45	8.28	11.98	13.14	12.96	13.65	13.00	13.91	13.26
20.xii.45	6.91	8.69	10.99	11.60	11.30	12.05	11.63	11.55
22.i.46	8.59	10.93	11.60	11.66	11.94	11.49	12.55	12.01
21.ii.46	8.24	9.93	10.94	10.97	11.45	10.45	11.08	11.34
24.iii.46	8.69	10.73	11.64	11.34	12.13	11.33	11.84	11.35
25.iv.46	8.88	10.48	11.16	11.26	12.89	12.29	13.34	12.94
11.vi.46	10.85	12.21	12.28	12.32	13.81	13.11	14.80	13.74
28.vii.46	9.14	10.08	10.84	11.01	13.84	10.96	11.30	11.10
3.ix.46	9.30	10.73	12.05	11.43	12.91	11.75	11.83	12.61
19.ix.46	8.49	10.71	11.11	11.53	12.55	12.51	10.99	12.20

Probability

Significant differences between means	0.05	0.01	0.001
within the table:	0.89	1.16	1.50 vol. O ₂ /100ml.

mean oxygen-carrying capacities of their bloods fell significantly ($P < 0.01$) and thereafter remained at approximately 75 per cent. of the level maintained throughout by the adequately supplemented groups. It is evident from Figure 1 and Table 2 that a supplement equivalent to 1 mg. Cu/day prevented the relatively rapid fall experienced by the individuals of group 1-Mu-0 and served to maintain the mean oxygen-carrying capacity at a level which rarely was significantly below that of the individuals which received 10 mg. Cu/day.

(e) *The Effect of Copper Deficiency on the Rate of Wool Production*

All of the experimental animals were shorn in October of 1945 and of 1946, and the total clean scoured wool produced by each individual was determined. From the findings set out in Table 3 there is no doubt that the amount of copper provided in the supplement was correlated with the amount of wool produced, up to a maximum which appeared to be reached when the animals received the equivalent of between 7.5 and 10 mg. Cu/day in addition to that ingested from the pastures. The asymptote of maximum response and its standard deviation were estimated in each series of observations by relating the annual production of clean scoured wool to the copper intake from the supplement, using for this the expression,

$$Y = k(1 - e^{-B(t+A)}),$$

in which Y = kg. clean scoured wool, t = the supplement in mg. Cu/day, k = the predicted maximum wool production, B and A being constants. The method of least squares was employed to derive the most likely values of k , B , and A and their standard deviations, the latter being based on 55 degrees of freedom. From analysis of variance, $k = 2.92 \pm 0.72$, $B = 0.215 \pm 0.059$, and $A = 5.44 \pm 1.52$ for the 1945 clip, and $k = 3.38 \pm 0.61$, $B = 0.368 \pm 0.073$, and $A = -2.45 \pm 0.565$ for the 1946 clip.

The differential equation of this expression, $dY/dt = B(k - Y)$, specifies that the increase in wool production per unit supplement of copper is proportional to the deficit of wool production below the maximum, k .

The curves obtained in this way are shown in Figure 2 in which the asymptote, k , for each set of data, is indicated with its S.D. plotted on either side. From the closeness of their fit to the observed means there is little doubt that the effect of the copper supplement was governed by the law of diminishing returns and that the maximum response was achieved when the supplement provided the equivalent of between 7.5 and 10 mg. Cu/day.

As the fodder conditions in 1946 were superior to those which prevailed in the previous year, and as the animals were by no means fully grown in 1945, the weight of wool produced in 1946 by all of the supplemented groups was considerably greater than that produced in 1945, notwithstanding the gradual worsening of the state of deficiency of all individuals that received less copper than was necessary to maintain them in copper equilibrium. The relationship between the weight of wool produced and the amount of copper in the supplement was not altered materially in the two clips — at each level of additional copper the proportional deficit from the asymptote remained, within the experimental error of the estimate, practically the same. The rate of wool production

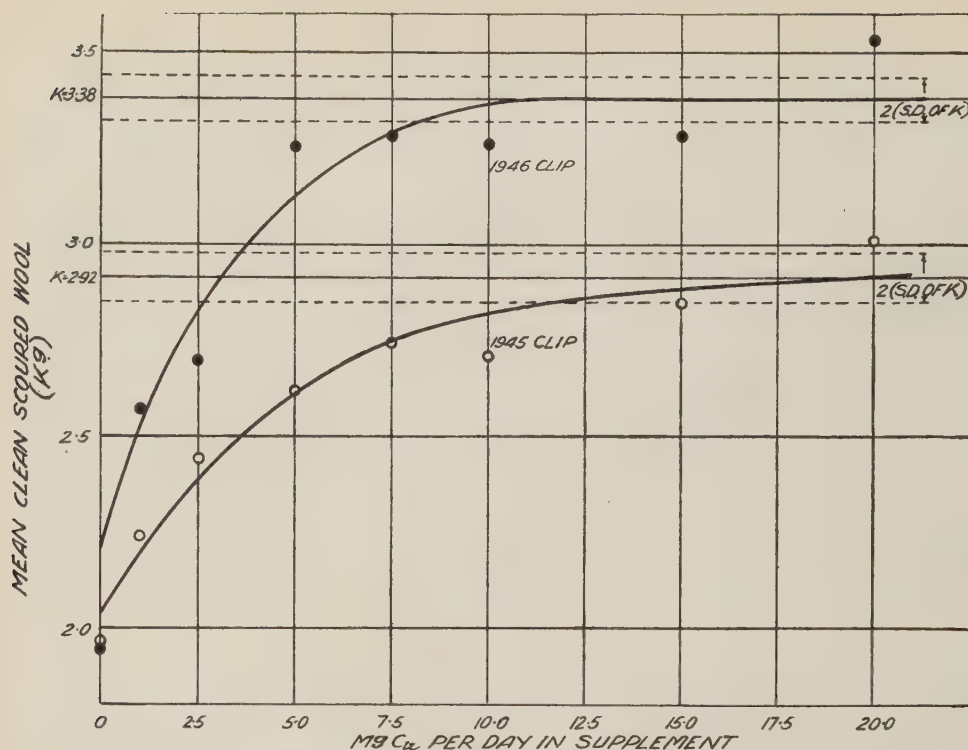


Fig. 2.—The influence of a graded series of degrees of copper deficiency on the annual production of clean scoured wool during the first two years of the experiment is shown. The observed group means are plotted in relation to curves (derived *vide text*) which specify that the increase in wool production per unit supplement of copper is proportional to the deficit of wool production below the maximum, k . The asymptote, k , is indicated with its S.D. plotted on either side. In each year the maximum response was achieved when the supplements provided the equivalent of between 7.5 and 10 mg. Cu/day.

(i.e. the quantity of wool produced per unit time) under these grazing conditions was the resultant of a number of interacting variables — the quantity and quality of the fodder derived from the pastures, the copper intake, the nature and extent of dietary factors which would complicate the absorption or otherwise deplete the copper reserves of the individual etc.—few of which may be evaluated with

EXPLANATION OF PLATE 1

The series of staples selected at random from each of the experimental groups illustrates changes in the appearance of the wool which supervene on copper deficiency. Both staples of each pair were drawn from the same part of the fleece, the first being grown in 1944 while the animals were depastured on sound country and the second in 1945 during the first year on the deficient terrain.

A range of degrees of deterioration is apparent in the first four pairs of staples which were taken from animals which received supplements (equivalent to 0, 1, 2.5, and 5 mg. Cu/day respectively) insufficient to maintain copper equilibrium. The supplements (equivalent to 7.5, 10, 15, and 20 mg. Cu/day respectively) administered to the sheep that grew the fleeces from which the second four pairs of staples were drawn were adequate to maintain a normal copper status and the process of keratinization was unaffected by the conditions of grazing in 1945.



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desirable precision in field experiments. A detailed analysis of minor differences in the copper supplement/fleece weight relationships which occurred in the two sets of observations is thus unlikely to yield cogent information.

As the extent of depression of the growth rate of the animals was similarly related to the degree of copper deficiency imposed, the effect on the rate of wool production may be ascribed to the partial failure of appetite rather than to specific effects on the physiological processes involved in wool production *per se*. In the absence of positive evidence to the contrary it may be concluded tentatively that the rate of wool production was limited by the supply of substrate (cf. Marston 1948) rather than by an impairment of the capacity of the cells in the follicles to utilize amino acids and to proliferate normally. The subsequent process of keratinization, however, is materially affected by copper deficiency (Marston 1946).

(f) *The Effect of Copper Deficiency on the Nature of the Wool*

Each of the experimental animals had the hereditary propensity to impress a well-defined crimp on its fleece — the flock had been selected, with this function as a criterion, from a strain noted for the character of its wool — and so any impairment of the physiological mechanism involved was readily discernible. The ability to impart crimp was lost very rapidly by the unsupplemented animals and, in all of those that received less copper than would maintain maximum efficiency, the degree of failure of this function was directly related to the extent of depletion of their copper reserves.

The changes in the appearance of the wool that supervened during the first year (1945) of grazing on the affected terrain are shown in Plate 1, in which the type of wool grown while the animals were depastured on sound country (1944) is contrasted with that produced in 1945. The staples were grown on the skin area defined in each animal by the bony protuberance of the acromion process of its right scapula and so are comparable. They were selected at random — the response within each group was remarkably constant.

The lesions that appeared in the fleeces grown by animals which received less than 7.5 mg. Cu/day — which amount was sufficient to maintain normal wool growth over the first year of observation — reflected well-defined stages of the range of deficiency states prevalent among flocks grazing on affected terrain in southern Australia.

The first sign of an impaired copper status is the tendency for the crimps to become less distinct (cf. the wool from R.770 which animal received 5 mg. Cu/day); secondary waves superimposed over the deteriorating crimps then appear (cf. R.821 which received 2.5 mg. Cu/day); and finally the ability to impart crimp is lost altogether (cf. R.787 and R.773 which received no copper and 1 mg. Cu/day respectively). In the second year of observation the condition of the fleeces produced by the animals which received 2.5 and 5 mg. Cu/day deteriorated further, and slight lesions became evident in some of the fleeces from the animals that received 7.5 mg. Cu/day, but the wool from the groups which received supplements containing 10 mg. Cu/day, or more, continued to be normally crimped. Obviously copper balance had been struck by supplements

which provided the equivalent of between 7.5 and 10 mg. Cu/day. This conclusion is identical with that derived from the quantitative considerations discussed in the previous section.

V. DISCUSSION

The rate at which the mean concentration of copper in the bloods of the unsupplemented group fell to a very low level implied a rapid depletion of the copper status under these grazing conditions. Equilibrium was established when between 7.5 and 10 mg. Cu/day were supplied in the supplement: above this level of intake no further improvement was apparent from the criteria employed to measure the physiological state of the animals, and below it the rate of depletion diminished progressively with each additional increment of copper provided in the supplements.

The supplements were administered in relatively concentrated solutions which might tend to favour reflex closure of the oesophageal groove and so lead the drench to by-pass the rumen and flow direct to lower levels of the intestinal tract. The fact that an evenly graded series of deficiency states supervened in the groups that received supplements containing suboptimum quantities of copper suggests, however, that the drenches entered the rumen to be diluted in its voluminous contents before passing to the intestine. It is inconceivable that the small quota of copper absorbed would be so directly related to the amount of copper provided in the drench if the latter flowed intermittently in relatively very high concentration over the absorption areas of the intestine.

The wool fleece was influenced profoundly by the state of copper deficiency induced, and both the rate of wool production and the deterioration of its quality were determined ultimately by the degree of depletion of the copper reserves. The decrease in the weight of wool produced by the copper-deficient animals was more likely the result of an integrated expression of the severity of the deficiency syndrome than of a direct influence of copper deficiency on the rate of cell division in the follicles, but the deterioration of the process of keratinization signified by the failure to impart crimp was certainly a specific effect of copper deficiency. During the course of depletion of the copper reserves this latter function is affected adversely long before there is any sign of an impairment of iron metabolism. The ability to impart normal crimp invariably failed when the blood-copper fell below 0.4 mg. Cu/l., even when the oxygen-carrying capacity was normal, and the extent of this failure was proportional to further decrease in blood-copper. Massive doses of iron exerted no beneficial effect when this function was impaired, but normal keratinization returned immediately when the blood-copper level was reinstated to its normal range by increasing the copper intake.

It is evident that the chemical processes responsible for keratinization depend fundamentally on copper.

VI. ACKNOWLEDGMENTS

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VII. REFERENCES

- DICK, A. T., and BINGLEY, J. B. (1947).—*Aust. J. Exp. Biol. Med. Sci.* **25**: 193-202.
- DICK, A. T., and BULL, L. B. (1945).—*Aust. Vet. J.* **21**: 70-2.
- FERGUSON, W. S., LEWIS, A. H., and WATSON, S. J. (1940).—*Jealott's Hill Res. Sta. Bull.* No. 1.
- FERGUSON, W. S., LEWIS, A. H., and WATSON, S. J. (1943).—*J. Agric. Sci.* **33**: 44-51.
- MARSTON, H. R. (1946).—"Nutrition and Wool Production." Symposium on Fibrous Proteins, published by the Society of Dyers and Colourists, Leeds, pp. 207-14.
- MARSTON, H. R. (1948).—*Aust. J. Sci. Res. B* **1**: 362-75.
- MARSTON, H. R., and LEE, H. J. (1948).—*J. Agric. Sci.* **38**: 216-21.
- MARSTON, H. R., LEE, H. J., and McDONALD, I. W. (1948*a*). *Ibid.* **38**: 222-8.
- MARSTON, H. R., LEE, H. J., and McDONALD, I. W. (1948*b*). *Ibid.* **38**: 229-41.

INACTIVATION OF GONADOTROPHINS

II. INACTIVATION OF PITUITARY AND CHORIONIC GONADOTROPHINS BY INFLUENZA VIRUS AND RECEPTOR-DESTROYING ENZYME

By W. K. WHITTEN*

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Summary

Chorionic gonadotrophin was rapidly inactivated by LEE-B influenza virus and receptor-destroying enzyme (*Vibrio cholerae*) preparations.

A crude anterior pituitary gonadotrophic preparation when incubated with LEE-B influenza or receptor-destroying enzyme preparations lost its ability to produce vaginal opening and increase in ovarian weight in immature rats. It was therefore concluded that the follicle-stimulating component of this preparation was destroyed but no deduction was drawn regarding the effect of luteinizing hormone.

I. INTRODUCTION

Up to the present time the action of influenza virus on blood-group and similar substances is the only direct enzymic action of a virus on a chemically defined substrate which has been described (Burnet 1947; deBurgh, Yu, Howe, and Bovarnick 1948). This action of influenza virus on such substances and on cell surfaces is almost completely paralleled by that of the receptor-destroying enzyme from *Vibrio cholerae* (Burnet loc. cit.; Burnet, McCrea, and Anderson 1947). In the first paper of this series (Whitten 1948) it was shown that preparations of both influenza virus and receptor-destroying enzyme inactivated serum gonadotrophin and it was therefore concluded that the enzymic mechanism which inactivated the hormone was the same as that which acted on the other mucoproteins. This conclusion was further supported by the similarity between blood-group substances and serum gonadotrophin which are both neutral mucoproteins containing N-acetylglucosamine and hexoses.

The investigation has been extended to include the effect of these enzymes on pituitary and chorionic gonadotrophins.

II. MATERIAL AND TECHNIQUE

Influenza Virus and Receptor-destroying Enzyme Preparations.—These preparations were prepared as previously described and were again supplied by Professor F. M. Burnet.

Chorionic Gonadotrophin.—This hormone was prepared from human pregnancy urine of high titre by the method described by Salmon and Hamblen (1943). In each test the amount used was in excess of that required to produce the maximal ovarian response. This procedure was adopted since no standard was available to determine the actual potency of the preparation.

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Pituitary Gonadotrophic Preparations.—Acetone-desiccated anterior pituitaries from geldings were extracted as described by Rinderknecht and Williams (1939) and fractionated as far as precipitation with 75 per cent. alcohol at pH 5.0. This precipitate was dried and each rat received the equivalent of 1 mg. of such a preparation, which produced ovaries weighing from 100-200 mg. The preparations thus contained both follicle-stimulating and luteinizing hormones.

Procedure for Incubation of Hormones with Enzymes.—The hormone preparations were dissolved in borate buffer, pH 7.0, and one-fifth of the volume of virus or enzyme preparation was added. The mixture was then incubated at 37°C. for three hours together with one drop of chloroform as a bacteriostatic. For each test a control duplicate was prepared in the same manner except that heat inactivated virus or enzyme was used. The solutions were then used for biological assay and were stored frozen during the test.

Hormone Assay.—The hormone assay technique was the same as previously used (Whitten loc. cit.) except that the animals received daily injections for four days and were slaughtered on the fifth day. Groups of ten rats received equal doses of the test or control preparations, and vaginal opening and ovarian weights were utilized as criteria for hormone action.

III. RESULTS AND DISCUSSION

The results of these observations are recorded in Table 1 and show that both the gonadotrophic preparations completely lost their ability to induce vaginal

TABLE 1

THE EFFECT OF INFLUENZA VIRUS AND RECEPTOR-DESTROYING ENZYME ON CHORIONIC AND PITUITARY GONADOTROPHIC PREPARATIONS

	Hormone	Enzyme	Mean Ovarian Wt. (mg. \pm s.e.)	No. of Vaginae Open
Test 1	Chorionic gonadotrophin	Influenza virus	17 \pm 1	0
	Chorionic gonadotrophin	Influenza virus (inactivated)	67 \pm 2	10
Test 2	Chorionic gonadotrophin	Receptor-destroying enzyme	15 \pm 1	0
	Chorionic gonadotrophin	Receptor-destroying enzyme (inactivated)	66 \pm 5	10
Test 3	Pituitary gonadotrophin	Influenza virus	17 \pm 1	0
	Pituitary gonadotrophin	Influenza virus (inactivated)	103 \pm 12	10
Test 4	Pituitary gonadotrophin	Receptor-destroying enzyme	16 \pm 1	0
	Pituitary gonadotrophin	Receptor-destroying enzyme (inactivated)	175 \pm 15	10

Ten rats were used for each group. Mean ovarian weight of untreated animals 16 \pm 1 mg.

opening or an increase in ovarian weight after incubation with active influenza virus or receptor-destroying enzyme. These findings are interpreted as a destruction of both chorionic gonadotrophin and follicle-stimulating hormone.

It is concluded that the latter hormone was inactivated, as it has been shown by Simpson, Hao Li, and Evans (1942) that pure luteinizing hormone produces only a small increase in ovarian weight even at high dose rates. However, in combination with follicle-stimulating hormone it augments the latter's action on the ovaries. Thus if a mixture of both hormones were to lose its capacity to cause an increase in ovarian weight the follicle-stimulating or both hormones must have been destroyed. Therefore it appears that receptor-destroying enzyme and influenza virus inactivated follicle-stimulating hormone but no conclusions can be drawn regarding its effect on luteinizing hormone. This latter aspect is at present under investigation.

These experiments and those described earlier show that serum gonadotrophin, chorionic gonadotrophin, and follicle-stimulating hormones are inactivated by influenza virus and receptor-destroying enzyme. It is suggested that these enzymes alter the structure of the hormones upon which depend the gonadotrophic action and that the same or similar structures are common to all three gonadotrophins.

The specific action of gonadotrophins on the gonad appears to be direct and for such action to take place it is probable that the hormone is adsorbed by the gonad. This view is supported by the observation of McPhail, Parkes, and White (1933) that the ovary withdraws gonadotrophins from the circulation and also by the findings of Seidlin (1940) and of Jungck, Heller, and Nelson (1947) that the ovary inactivates the hormone. Since other mucoproteins have been shown to participate in specific adsorptions some of which can be prevented by receptor-destroying enzymes, one might postulate that the inactivation of gonadotrophins by these enzymes is due to an alteration of the hormones which prevents their adsorption by the gonad.

IV. ACKNOWLEDGMENTS

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I am again indebted to Professor F. M. Burnet, F.R.S., for enzyme and virus preparations and for his helpful suggestions during this investigation. I also wish to thank Dr. A. Bolliger for valuable advice and for providing laboratory facilities, and Dr. W. P. Rogers for his criticism.

V. REFERENCES

- DEBURGH, P. M., YU, P. C., HOWE, C., and BOVARNICK, M. (1948).—*J. Exp. Med.* 87: 1-10.
BURNET, F. M. (1947).—*Aust. J. Sci.* 10: 21.
BURNET, F. M., MCCREA, J. F., and ANDERSON, S. G. (1947).—*Nature* 160: 404-5.
JUNGCK, E. C., HELLER, C. G., and NELSON, O. (1947).—*Proc. Soc. Exp. Biol. N.Y.* 65: 148-52.
MCPHAIL, M. K., PARKES, A. S., and WHITE, W. E. (1933).—*J. Physiol.* 79: 180-4.
RINDERKNECHT, H., and WILLIAMS, P. C. (1939).—*J. Endocrinol.* 1: 117-27.
SALMON, A. A., and HAMBLE, E. C. (1943).—*Endocrinology* 33: 257-9.
SEIDLIN, S. M. (1940).—*Ibid.* 26: 696-702.
SIMPSON, M. E., HAO LI, C., and EVANS, H. M. (1942).—*Ibid.* 30: 977-84.
WHITTEN, W. K. (1948).—*Aust. J. Sci. Res. B* 1: 271-7.

THE ANATOMY OF THE TIMBERS OF THE SOUTH-WEST PACIFIC AREA

I. ANACARDIACEAE

By H. E. DADSWELL* and H. D. INGLE*

(Plates 1-6)

[*Manuscript received August 17, 1948*]

Summary

The results reported are the first of a series dealing with the timbers of the south-west Pacific area and cover 22 genera of the family Anacardiaceae. Based on the examination of available species, the anatomical features of each genus have been summarized and the results set out in tabular form for comparative purposes. The relationship between botanical classification and the wood anatomy of the genera concerned has been discussed. From the information presented it is suggested that a simple card sorting key can be readily developed.

I. INTRODUCTION

A survey of the anatomical characteristics of a particular family should preferably include examples from as wide a geographical range as possible. Many papers have been published from time to time dealing with the timbers of various families and these have taken into consideration available material from all parts of the world.

In Australia, the work of this nature has previously been restricted to the Australian representatives, but in recent years it has been necessary to extend such surveys to timbers of New Guinea, the British Solomon Islands, and other islands of Polynesia. However, experience has shown that, in addition, reference should be made to specimens of the family under consideration occurring somewhat further afield, e.g. in Malaya, the East Indies, and the Philippines, the reason being that a large number of tropical genera, from which commercial timber may be derived, are distributed throughout these areas as well as in Australia, New Guinea, and Polynesia. Merrill (1946) has instanced several examples of the attenuated distribution of certain genera in these regions. Therefore, in the work to be described, of which this paper forms the first part, attention has been paid to the timber species of the various families investigated which occur in the wider sphere of Malaysia,† Australia, New Guinea, and Polynesia. These regions form a very natural geographical and botanical unit and will, for the purpose of this work, be referred to generally as the south-west

* Division of Forest Products, C.S.I.R.

† Covering Malaya, the various islands of the East Indies, and the Philippines.

Pacific area. It is not inappropriate, therefore, for a detailed study to be made of the anatomy of the timber species of this area, to be carried out in a manner similar to that followed by Record and Hess (1943) in their work on the "Timbers of the New World."

The importance of knowledge of the timbers of the south-west Pacific area became apparent during the second world war when bases were established in various Pacific areas. Local timbers were used wherever possible and, as a result, there was a considerable demand for information on identification and properties which was not at that time readily available. Latterly there has been an increase in the importation into Australia of timbers from Malaya, Borneo, New Guinea, and certain Pacific islands. This has necessitated examination of the anatomy of these timbers, which examination is being carried out one family at a time.

In such investigations it is desirable wherever possible to examine the timbers of all the available species of a particular family represented in the area under consideration. The recording of anatomical details of each species is, however, not always essential and some wood anatomists have of recent years adopted the practice of describing the main anatomical features of the genus, only referring to specific differences where such are outstanding. This practice has no doubt arisen because of the desire to record generic differences as an aid to identification and because, in many cases, specific differences are minor. From the anatomical aspect it is often relatively simple to identify the genus but more difficult to determine the species without the examination of a much wider range of material than is usually available. For these reasons in the present work generic summaries only have been recorded.

II. GENERAL

The family Anacardiaceae has a wide distribution but is most abundantly represented in tropical and semi-tropical areas. The woods of the genera that occur in the New World (North and South America and the islands of the West Indies) have been described by Record and Hess (1943). Hess has more recently (1946) published details of the anatomy of nineteen of the genera of this region and he has developed a dichotomous key for the identification of these genera. From these works it is apparent that many of the timbers have found commercial application.

Some 22 genera of the family are represented in the south-west Pacific area; of these only four, namely, *Rhus*, *Camposperma*, *Pistacia*, and *Spondias*, also occur in the New World. Many of the genera include species growing to trees of medium or large size from which commercial timber is obtainable. In some instances the timbers are known favourably in world trade circles, a particular example being those of the genus *Dracontomelum*. Published information is available on a number of timbers of this family and it is apparent that these timbers have been used to a greater or lesser extent in India, Burma, Malaya, Sumatra, Java, and the Philippines. The works of Pearson and Brown (1932)

covering Indian timbers, Desch (1941) Malayan timbers, Janssonius (1908) timbers of Java, Cockrell (1941) timbers of Sumatra, and Reyes (1938) Philippine timbers were freely consulted in the present investigation which has been directed more at developing a knowledge of the genera and their interrelationships than at detailed descriptions of individual species.

The general distribution of the family in the south-west Pacific area is shown in Figure 1; from this it is apparent that the family is well represented in New Guinea, the Solomon Islands, and Australia as well as in the Malaysian region. It should not be assumed that the distribution as shown is complete; it is almost certain that a wider spread of various genera will be found when the forest areas of the region are better known botanically. The commercial importance of the timbers will grow as they become better known; a great many of the species of various genera are used for a variety of purposes locally. In this respect the representatives of the family in the south-west Pacific area seem to have greater possibilities than those of the New World, due perhaps to the greater average size of the trees and to their greater abundance.



Fig. 1.—Distribution of genera examined in the south-west Pacific area.

In all the literature on this family, reference is made to the poisonous qualities of the sap; the exudations both from the wood and the zone between bark and wood have decidedly irritant properties and give rise to skin lesions. Thus the handling of the logs for sawing is most difficult and this accounts in no

small measure for the fact that otherwise excellent timber trees are left standing. Desch (1941) records that infection may result from contact with leaves, bark, or "green" wood. Species of the following genera, *Buchanania*, *Gluta*, *Melanorrhoea*, *Melanochyla*, *Rhus*, *Swintonia*, and *Semecarpus* have been particularly mentioned by Burkill (1935) in this regard, and these irritant properties were noticed with the species of *Semecarpus* occurring in New Guinea. It is probable that these irritant properties are due to the presence of an acid and/or phenol present in the resinous exudate (also called resin, gum, or latex) from many parts of the tree. It is well known that in this family horizontal gum canals are particularly common in the rays. Lane-Poole (1925) has recorded that large quantities of an oil were collected from the wood of a species of the genus *Pentaspadon*.

A large number of the trees of different species of this family develop reaction wood. This statement is made because of the fairly common occurrence of tension wood identified by the presence of gelatinous fibres and by the extremely woolly sawcut. The Australian species *Euroschinus falcatus* Hook. is a very good example of this and it has been reported by Boas (1947) as difficult to finish owing to its woolly grain, and to be tough and hard to cut with a chisel. These features are typical of tension wood and there is no other reason for hardness in such a low density wood (air-dry density ranging from 25-35 lb./cu.ft.). Desch (1941), too, in discussing the sawing properties and workability of various species of *Mangifera*, *Swintonia*, *Parishia*, and *Camptosperma*, refers to the extreme woolliness of the sawcuts and to the fact that the saw often jammed when cutting up particular logs. These features are indicative of the presence of considerable amounts of tension wood.

Material for this survey was taken from the standard collection of the Division of Forest Products. This includes various authenticated specimens received on an exchange basis from forestry authorities in India, Burma, the Dutch East Indies, and the Philippines. Special reference, however, must be made to the specimens of Malayan timbers received in 1941 through the courtesy of Dr. H. E. Desch; to specimens of New Guinea timbers, kindly made available by Mr. C. E. Lane-Poole, collected during his survey in that region in 1924; to specimens from the Waterhouse collection in New Guinea and the Solomon Islands supplied by the late Dean S. J. Record of the Yale School of Forestry (see Mary Record 1945); to specimens of timbers from New Guinea and surrounding islands and from North Borneo collected by the Australian Army Forestry Units during the Pacific war period; to specimens from New Guinea supplied by Mr. J. B. McAdam, Acting Secretary of the Forests Department of the Papua-New Guinea Administration; and to specimens from the British Solomon Islands collected by Mr. F. S. Walker in 1945 and 1946. The botanical identifications of the material from New Guinea, collected by Mr. Lane-Poole and by the Australian Army, and of that from the British Solomon Islands collected by Mr. F. S. Walker were carried out by Mr. C. T. White, Government Botanist, Brisbane.

III. THE ANATOMICAL CHARACTERISTICS OF THE GENERA EXAMINED

For each genus the anatomical details for the various species examined have been summarized in the one brief description. Note has, in each case, been taken of information published on particular species and where the present examination has indicated some divergences these have been pointed out. As additional authentic material becomes available for detailed examination some slight modification of these generic summaries may be necessary, but it is not expected that changes will be more than minor, as, generally speaking, the structure is very uniform within each genus. In addition to the verbal description of the genera, certain important anatomical features have been tabulated (see Table 1) and some grouping has been attempted (see Table 2).

No key to the identification of the genera examined has been included, but from the descriptions and the data given in Tables 1 and 2, a card-sorting key for the genera could readily be prepared.

(a) *Blepharocarya*

(i) *General*.—This is a monotypic genus confined to north Queensland, Australia. The tree is small to medium sized, up to 90 ft. high and 3 ft. in diameter with a more or less cylindrical trunk and a slightly buttressed base. Truewood light pinkish-brown at times with greenish tints; generally straight grained and somewhat lustrous; moderately light in weight and easy to work. Used chiefly for tallow cask staves and brush stocks; possible uses: furniture and interior finish.

(ii) *Structure*.—*Growth rings* indistinct. *Vessels* visible to the naked eye, the largest averaging 230μ in tangential diameter; solitary, varying from 40-50 per cent., and in radial multiples of 2-4, occasionally up to 5; evenly distributed: vessel elements usually truncated; perforation plates simple, usually horizontal to occasionally obliquely inclined; inter-vessel pitting bordered, alternate, apertures lenticular, almost horizontally inclined, borders $7-10\mu$ in diameter. *Rays* mainly biseriate, occasionally uniseriate, up to 30 cells high, heterogeneous with 2-3 rows of upright cells, crystals* sparse in upright cells; vessel-ray pitting half-bordered, the apertures rounded or irregular to elongated; gum canals absent. *Parenchyma* sparse, paratracheal incompletely vasicentric, crystals absent. *Fibres* non-septate, very sparsely septate in some samples, pitting fine and inconspicuous, indistinctly bordered, walls of fibres usually thin.

(iii) *Material*.—*Blepharocarya involucrigeria* F.v.M., 6 samples, Queensland, Australia.

(b) *Bouea*

(i) *General*.—This genus is represented by 4 species occurring in Malaya, Burma, Java, and the Moluccas. Only one species was available for study. Generally small trees with edible fruit. Truewood pale brown with a pinkish

* It has been generally assumed by wood anatomists that the crystals observed in the cells of this and other families are of calcium oxalate. Work on the nature and identification of the crystal inclusions in various timbers is at present in hand.

tinge, somewhat lustrous, generally straight grained, without odour or taste. Moderately light in weight to moderately heavy. Used locally for house posts and general construction.

(ii) *Structure*.—*Growth rings* not evident macroscopically. *Vessels* distinct to the naked eye, evenly distributed, up to 50 per cent. solitary, generally rounded, remainder in radial multiples of 2-3; maximum tangential diameter $215\ \mu$; perforation plates simple, usually horizontal; inter-vessel pitting bordered alternate, apertures lenticular, almost horizontal, borders crowded $9-12\ \mu$ in diameter; tyloses uncommon. *Rays* uniseriate to biseriate, moderately high, up to 40 cells; heterogeneous, with 1-2 rows of upright cells containing crystals; vessel-ray pitting half-bordered, the apertures large, rounded to irregular in shape, sometimes gash-like; gum canals absent. *Parenchyma* abundant, paratracheal, vasicentric to occasionally aliform, and apotracheal in regularly to irregularly spaced bands up to 12 cells wide, usually 4-8; resinous contents abundant; crystals lacking. *Fibres* non-septate, with moderately thick to fairly thin walls; pits small and inconspicuously bordered.

(iii) *Material*.—*Bouea macrophylla* Griff., 10 samples Malaya.

(c) *Buchanania*

(i) *General*.—A genus of approximately 20 species of trees or shrubs occurring throughout south-eastern Asia, Malaysia, New Guinea, Solomon Islands, Queensland, and northern Australia. Material representing 7 tree species was available for study. Tree species mainly small to medium sized, usually less than 60 ft. overall; buttresses usually absent but if present small. Truewood generally pale grey to pinkish-brown, usually somewhat lustrous; soft to moderately soft, generally fine textured, and straight grained; not durable.

(ii) *Structure*.—*Growth rings* inconspicuous. *Vessels* generally few ($2-4/\text{sq. mm.}$) evenly distributed, visible to the naked eye; solitary pores varying from 30-60 per cent. in the different species, remainder of pores in radial multiples of 2-3 occasionally up to 4; clusters few; maximum tangential diameters from $210-290\ \mu$; perforation plates exclusively simple; inter-vessel pitting moderately coarse, bordered, alternate, apertures lenticular, included; borders circular, $10-12\ \mu$ in diameter; tyloses few to absent. *Rays* 2-3 seriate, up to 45 cells high, heterogeneous with 1-2 rows of upright cells; crystals not observed; horizontal gum canals present in all species examined, visible on clean tangential surface with lens, thin-walled epithelial lining distinct microscopically; vessel-ray pitting half-bordered, the apertures large, rounded to irregular or elongated in shape. *Parenchyma* not abundant, paratracheal vasicentric to weakly aliform in a few samples, not evident macroscopically; crystals not observed. *Fibres* thin walled, non-septate; pitting inconspicuous and indistinctly bordered.

(iii) *Material*.—*Buchanania heterophylla* K. Schum., 3 samples New Guinea; *B. lucida* Bl., 3 samples Malaya; *B. macrocarpa* Lauterb., 1 sample New Guinea; *B. mollis* Lauterb., 1 sample New Guinea; *B. muelleri* Engl., 8 samples Northern Territory, Australia; *B. sessifolia* Bl., 4 samples Malaya; *B. solomonensis* Merr. & Perry, 2 samples Solomon Islands.

(d) *Campnosperma*

(i) *General*.—A genus of approximately 10 species occurring in tropical regions of the world. Hess (1946) mentions 2 species (*C. gummifera* L. and *C. panamensis* Standl.) as occurring in swampy lands of the lower Amazon and Atlantic coastal areas of southern Central America. The other species are found in Malaysia, New Guinea, and Solomon Islands; material from 5 species has been examined. These species are of present or potential commercial importance, the 3 Malayan species supplying veneer for match splints and also sawn case timber. The New Guinea and Solomon Islands' species are medium to moderately large sized trees up to 120 ft. overall height with prominently buttressed trunks up to 2 ft. in diameter. The truewood of all species is somewhat similar, pale grey-brown to pinkish-brown, the sapwood being ill-defined from the truewood; moderately light in weight, of fine uniform texture, but sometimes interlocked in the grain, lustre low; rays generally darker and prominent on split radial face.

(ii) *Structure*.—*Growth rings* not evident macroscopically. *Vessels* small and indistinct to the naked eye, numerous (over 20/sq.mm.), evenly distributed, varying from 35-60 per cent. solitary, some in radial multiples of 2-3; maximum tangential diameters 125-160 μ ; perforation plates both simple and scalariform, the latter with numerous fine bars (Plate 4, Fig. 2); inter-vessel pitting fairly coarse, bordered, alternate; apertures horizontal, lenticular to slit-like, included; borders 9-14 μ diameter; tyloses generally few. *Rays* 1-3 cells wide, up to 45 cells high, heterogeneous with 2 or 3 rows of upright or squarish cells; crystals absent; a few multiseriate containing one or sometimes two horizontal gum canals, surrounded by 2 or 3 layers of epithelial cells, observed in all species examined but sometimes absent from a particular sample; resinous deposits abundant in ray cells; vessel-ray pitting half-bordered, characteristically gash-like to scalariform. *Parenchyma* generally absent but there may be occasional paratracheal cells. *Fibres* medium to thin walled, septate in part but these sparse and sporadic; pitting simple or indistinctly bordered.

(iii) *Material*.—*Campnosperma auriculata* (Bl.) Hook. f., 1 sample New Guinea; *C. brevipetiolata* Volkens, 7 samples New Guinea and Solomon Islands; *C. macrophylla* Hook. f., 2 samples Malaya; *C. minor* Corner, 1 sample Malaya; *C. wallichii* King, 7 samples Malaya.

(e) *Dracontomelum*

(i) *General*.—A genus of 6 species occurring in Malaysia, New Guinea, and also in Fiji; 4 species are of commercial importance — *D. dao*, *D. sylvestre*, *D. edule* from the Philippines, and *D. mangiferum* (sengkuang, Malaya; laup or New Guinea walnut), occurring throughout Malaysia and New Guinea. All are medium sized to large trees with more or less pronounced buttresses. The truewood varies from light brown, greyish or greenish-yellow, to reddish-brown, often streaked with varying amounts of deep brown or almost black; often resembling true walnut (*Juglans* spp.); sapwood sometimes distinct from the truewood, generally pinkish or greyish-yellow; texture moderately fine to coarse;

straight grained or interlocked; moderately light to moderately heavy. Generally used for veneers, furniture, and cabinet making, and also for light construction and interior finish.

(ii) *Structure*.—*Growth rings* usually not distinguishable. *Vessels* distinct to the naked eye, generally few (1-3/sq.mm.) evenly distributed, up to 45 per cent. solitary, tending to be somewhat radially flattened in cross section, remainder in radial multiples of 2-3 with occasional multiples of smaller pores up to 6; maximum tangential diameters from 290-305 μ ; perforation plates exclusively simple; inter-vessel pitting coarse, bordered, alternate, the apertures generally lenticular, horizontal, and included, diameter of borders 14-18 μ ; tyloses characteristically abundant. *Rays* 2-5 seriate, majority 3-5, and up to 30 cells high, heterogeneous with 1 or 2 rows of upright or squarish cells; crystals abundant in marginal and procumbent cells; horizontal gum canals not observed in any samples examined; vessel-ray pitting large, half-bordered, the apertures rounded to irregular and sometimes elongated in shape with narrow border. *Parenchyma* paratracheal vasicentric, aliform to confluent; in *D. mangiferum* wide bands which may have been apotracheal were observed in one or two samples; crystals observed in a few samples. *Fibres* moderately thin walled, exclusively septate, the septa extremely fine; pitting inconspicuous, simple.

(iii) *Material*.—*Dracontomelum dao* (Blanco) Merr. and Rolfe, 1 sample Philippines; *D. mangiferum* Bl., 7 samples New Guinea and Malaya.

(f) *Euroschinus*

(i) *General*.—A genus consisting of 5 species occurring in eastern Australia, New Guinea, and New Caledonia. Medium to large trees 60-110 ft. in height with diameters of 2-3 ft. having small buttresses at the base of the stem. The truewood is generally pinkish-brown, frequently with yellowish or greenish streaks; light to moderately light in weight, texture fine and uniform; moderately fissile.

(ii) *Structure*.—*Growth rings* not marked. *Vessels* small and indistinct to the naked eye, rather numerous in *E. papuanus* (15-20/sq.mm.), generally in radial multiples of 2-4, sometimes up to 6, occasionally in clusters, 20-30 per cent. solitary, tangential diameter of largest 140-180 μ ; inter-vessel pitting moderately coarse, bordered, alternate, apertures lenticular, borders 10-15 μ in diameter; perforation plates simple, although a few scattered reticulate plates were found in 3 samples of *E. falcatus* (Plate 4, Fig. 1); tyloses infrequent. *Rays* 1-4 cells wide, generally 2-3; up to 30 cells high; heterogeneous with 1-4 rows of upright or squarish cells containing crystals, sometimes chambered; crystals occasionally present in procumbent cells; resinous deposits moderately abundant; horizontal gum canals present, sometimes large and distinct to naked eye in *E. papuanus*; vessel-ray pitting half-bordered, the apertures irregular in shape, often scalari-form. *Parenchyma* paratracheal, usually sparse, sometimes vasicentric to very sparsely aliform in *E. papuanus*. *Fibres* moderately thin walled, septate fibres usually absent to sporadic in vicinity of vessels; pitting inconspicuous, small, and indistinctly bordered.

(iii) *Material*.—*Euroschinus falcatus* Hook., 10 samples Queensland; *E. papuanus* Merr. and Perry (mspt.), 5 samples New Guinea.

(g) *Gluta*

(i) *General*.—A genus of some 6 tree species occurring from southern India through Burma to western Malaysia.* *G. travancorica* Bedd., occurring in Travancore, is a large tree of some commercial importance. *G. tavoyana* Hook. f. occurs in Burma. Three species, *G. elegans* Kurz, *G. reinghas* L., and *G. velutina*, occur in Malaya, Sumatra, Java, and Borneo; the wood of these three species is common with the wood from another genus (*Melanorrhoea*) is known as "rengas." The trees vary from moderately small (60 ft.) to large and up to 120 ft. in height. The truewood of all species yields some handsome timber of a deep red to reddish-brown colour, often streaked with darker bands; the extent of this dark red wood appears to vary between species and between particular samples. The sapwood is often wide, up to 7 in. and light coloured. The timbers are moderately heavy, fairly coarse textured and generally somewhat interlocked.

(ii) *Structure*.—*Growth rings* often simulated by bands of resin-filled fibres, and/or by bands of apotracheal parenchyma. *Vessels* few to moderately abundant, variable in size, the largest conspicuous to the naked eye, majority solitary (up to 75 per cent.) with a few radial multiples of 2-3, occasionally up to 4; distribution even; maximum tangential diameters up to 310 μ ; perforation plates exclusively simple; inter-vessel pitting moderately coarse, bordered, alternate, apertures lenticular, borders 9 μ in diameter; tyloses usually abundant. *Rays* generally uniseriate, or occasionally biseriate in part; homogeneous to weakly heterogeneous; up to 25 cells high; horizontal gum canals present, the rays at this point being 3-4 cells wide; resinous deposits common; silica deposits present in species examined and reported for *G. tavoyana* and *G. travancorica* by Pearson and Brown (1932); crystals absent; vessel-ray pitting half-bordered, the apertures rounded to elongated. *Parenchyma* paratracheal, sparse, usually incompletely vasicentric; apotracheal in irregularly spaced bands 2-6 cells wide, often discontinuous; resinous deposits abundant; crystals not observed, Pearson and Brown (1932) mention occasional siliceous inclusions similar to those found in the ray cells, but these were not observed in material examined. *Fibres* moderately thick walled, non-septate; pits inconspicuous and indistinctly bordered; resinous deposits abundant in some, these appearing in transverse section as narrow to wide dark bands.

(iii) *Material*.—*Gluta reinghas* L., 1 sample Dutch E. Indies.

(h) *Koordersiodendron*

(i) *General*.—This is a genus occurring in the Philippines, Borneo, Celebes, and Halmahera. A medium to large sized tree, with a bole up to 50 ft. long with small buttresses. Truewood reddish-brown, sapwood pale pink and distinct from the truewood; heavy to moderately heavy, grain often interlocked, texture

* Malaya, Java, Sumatra, Borneo, and adjacent smaller islands.

medium, somewhat lustrous. Said to be durable to some extent out of doors, but used mainly for flooring, house construction, furniture, and cabinet making (Reyes 1938).

(ii) *Structure*.—*Growth rings* absent or indistinct. *Vessels* visible to the naked eye and moderately numerous (4-8/sq.mm.), evenly distributed, up to 60 per cent. solitary, remainder in short radial multiples of 2-3; maximum tangential diameter 230 μ ; perforation plates exclusively simple; inter-vessel pitting moderately coarse, bordered, alternate; apertures lenticular; borders 12-14 μ in diameter; tyloses characteristically abundant. *Rays* 1-3 seriate, up to 30 cells high, heterogeneous with 1 to several rows of upright cells containing crystals which are characteristic, being contained in chambered cells, frequently interspersed with tall upright cells without crystals (Plate 6, Fig. 4); resinous deposits abundant; horizontal gum canals present and filled with deposits, rather small; vessel-ray pitting large, half-bordered, the apertures rounded to elongated. *Parenchyma* paratracheal, generally incompletely vasicentric, inconspicuous macroscopically. *Fibres* moderately thick walled, septate; pits simple and inconspicuous.

(iii) *Material*.—*Koordersiodendron pinnatum* (Blanco) Merr., 3 samples Philippine Is. and North Borneo; *Koordersiodendron* sp., 1 sample Halmahera.

(i) *Mangifera*

(i) *General*.—A genus of some 30 species including 8 species which are described as timber trees, occurring in India, Burma, Malaysia, New Guinea, and Solomon Islands; generally medium to large with small buttresses. Truewood greyish to reddish-brown, often streaked with darker bands, sapwood generally wide and sometimes distinct from the truewood; varying from moderately heavy to heavy; texture fairly fine, grain sometimes interlocked; not durable, used mainly for interior finish, furniture, and cabinet work. Samples from 4 of these species were available for study. *Mangifera indica* is perhaps more valuable for its fruit than its timber, and is now widely cultivated in many parts of the tropics.

(ii) *Structure*.—*Growth rings* sometimes marked by denser bands of fibres and sometimes indicated by narrow to moderately wide bands of parenchyma, though this is seldom a constant feature. *Vessels* indistinct to visible to the naked eye, moderately numerous and evenly distributed, sometimes up to 45 per cent. solitary; remainder in radial multiples of 2-3, occasionally up to 5, and sometimes in clusters; maximum tangential diameters ranging from 150-230 μ ; perforation plates exclusively simple, horizontal to oblique; inter-vessel pitting fairly coarse, alternate, apertures lenticular, borders 10-14 μ in diameter. *Rays* uniseriate in *M. solomonensis* and in *M. altissima* (Reyes 1938), usually 1-2 seriate, occasionally up to 3 seriate in the remainder; low but sometimes up to 25 cells high; heterogeneous with 1-2 rows of upright cells, crystals abundant in marginal and procumbent cells; horizontal gum canals not observed and reported to be absent by other investigators; vessel-ray pitting large, half-bordered, the apertures rounded to irregular in shape. *Parenchyma* variable, generally paratracheal, vasicentric in a wide sheath to aliform, occasionally to frequently confluent in all

species; in some species apotracheal parenchyma is developed to varying degrees, frequently as apparently terminal bands 2-6 cells wide in *M. solomonensis*, *M. indica*, and *M. altissima*, but sometimes only 1 cell wide and sporadic in occurrence. *Fibres* moderately thick walled, non-septate, pitting indistinctly bordered, resinous deposits common in some samples.

(iii) *Material*.—*Mangifera altissima* Blanco, 1 sample Philippine Islands; *M. minor* Bl., 6 samples New Guinea and Solomon Islands; *M. indica* L., 1 sample India; *M. solomonensis* C. T. White, 2 samples Solomon Islands.

(j) *Melanochyla*

(i) *General*.—A genus of some 6 species confined apparently to Malaya; samples from 2 species were available for study. Medium to large sized trees. The truewood is generally dark brown with darker almost black streaks. The sapwood is generally differentiated from the truewood and 3-6 in. in width, generally pale yellow-brown with a pink or green tinge. The timbers are moderately heavy to heavy; grain generally interlocked and sometimes wavy: texture moderately coarse; generally not used commercially, probably because of the irritant sap (Desch 1941).

(ii) *Structure*.—*Growth rings* not evident. *Vessels* moderately few (3-5/sq. mm.), evenly distributed, 45-50 per cent. solitary, remainder in radial multiples of 2-3; maximum tangential diameter 170-210 μ ; perforation plates exclusively simple; inter-vessel pitting only moderately coarse, alternate, the apertures lenticular, borders crowded, 8-12 μ in diameter; tyloses common. *Rays* 1-2 seriate, usually low, up to 20 cells high, heterogeneous with one or two rows of upright cells; crystals common in procumbent cells in *M. kunstleri*, absent in *M. bracteata*; horizontal gum canals present; vessel-ray pitting half-bordered, the apertures rounded to irregular in shape; resinous deposits common. *Parenchyma* paratracheal, aliform to occasionally confluent; resinous deposits fairly common. *Fibres* non-septate, moderately thick walled, pits indistinctly bordered.

(iii) *Material*.—*Melanochyla kunstleri* King, 1 sample Malaya; *M. bracteata* King, 1 sample Malaya.

(k) *Melanorrhoea*

(i) *General*.—A genus consisting of some 7 or more species of trees occurring from Burma and Indo-China to Malaya, Sumatra, Borneo, and New Guinea. They are generally medium sized to moderately large trees, 80-100 ft. high; collectively known as "rengas" in Malaya. Two species, *M. usitata* Wall. in Burma and *M. laccifera* Pierre in Indo-China, are important commercial sources of lacquer. The truewood is generally a bright orange-red, which darkens on exposure to a deep mahogany-red, and shows streaks of darker purplish or deep brown colour simulating growth rings on longitudinal faces; the sapwood is pale cream or sometimes pinkish, distinct from the truewood and is usually wider in smaller trees. The timbers are moderately heavy to heavy, varying in density from 35 to almost 60 lb./cu.ft. air-dry; grain shallowly interlocked, texture somewhat fine

and even, the wood taking a smooth finish. The truewood is said to be moderately durable but the main uses of these timbers are in the joinery, furniture, and cabinet-making fields.

(ii) *Structure*.—*Growth rings* sometimes simulated macroscopically by darker bands of fibres. *Vessels* indistinct to large and distinct to the naked eye, evenly distributed, moderately few in number (2-4/sq.mm.); maximum tangential diameters varying from 210-310 μ ; solitary pores numerous, varying from 25-60 per cent., remainder generally in radial multiples of 2-3, occasionally up to 6; perforation plates exclusively simple; inter-vessel pitting alternate, apertures lenticular, borders varying from 8-14 μ in diameter; tyloses abundant. *Rays* in majority of samples uniseriate, sometimes biseriate in part, practically homogeneous, no true upright cells, crystals absent; silica inclusions present in all specimens examined; resinous deposits abundant; horizontal gum canals present in all species in rays 3-4 cells wide; vessel-ray pitting half-bordered, the apertures rounded to irregular in shape. *Parenchyma* paratracheal, usually sparse to occasionally vasicentric, chiefly in apotracheal bands, numerous, but irregularly spaced, sometimes discontinuous, 2-8 cells wide. *Fibres* moderately thick walled, non-septate, frequently filled with resin or having numerous resin plugs, usually alternating in bands with non-filled fibres; pitting indistinctly bordered.

(iii) *Material*.—*Melanorrhoea aptera* King, 2 samples Malaya; *M. laccifera* Pierre, 1 sample Indo-China; *M. pilosa* Lecomte, 2 samples Malaya; *M. torquata* King, 2 samples Malaya; *M. wallichii* Hook. f., 2 samples Malaya; *Melanorrhoea* spp., 5 samples Malaya, 1 sample Borneo, 2 samples New Guinea.

(l) *Microstemon*

(i) *General*.—A small genus of 2 or 3 species recorded from Malaysia; samples of only one species were available for study; large trees, but apparently not of commercial importance. Truewood yellowish-brown with faint pinkish tinge; moderately light, texture fine to medium, rather straight grained, somewhat lustrous.

(ii) *Structure*.—*Growth rings* not evident. *Vessels* visible to the naked eye, moderately numerous (6-10/sq.mm.), evenly distributed, 45-50 per cent. solitary, remainder in radial multiples of 2 and sometimes 3; maximum tangential diameter 200 μ ; perforation plates exclusively simple; inter-vessel pitting alternate, apertures lenticular, borders 10-12 μ in diameter; tyloses infrequent. *Rays* 2-4 seriate, up to 35 cells high, very markedly heterogeneous, with several rows of marginal and interspersed upright cells containing crystals (Plate 5, Fig. 1); horizontal gum canals present, small to moderately large; resinous deposits infrequent; vessel-ray pitting half-bordered, the apertures rounded to irregular or elongated. *Parenchyma* sparse paratracheal. *Fibres* fairly thin walled, septate, pits indistinct.

(iii) *Material*.—*Microstemon velutina* Engl., 3 samples Malaya.

(m) *Odina*

(i) *General*.—A genus of some 30 species of small or large trees mainly occurring in Africa from Sierra Leone to Abyssinia. Two species are recorded

from the area under consideration, *O. wodier* Roxb. widely distributed in India and Burma and *O. speciosa* Bl. from New Guinea. Truewood light red to brownish-red, sapwood pale white or yellowish-white and usually wide; moderately heavy and generally interlocked, texture moderately fine and even. The timber is only of local commercial importance.

(ii) *Structure*.—*Growth rings* not evident. *Vessels* visible to the naked eye, evenly distributed, moderately numerous (8-9/sq.mm.), up to 40 per cent. solitary, remainder in radial multiples, generally of 2-3; maximum tangential diameter 200 μ ; perforation plates simple; inter-vessel pitting moderately coarse, alternate, apertures lenticular, included, borders 12 μ in diameter; vessels of truewood invariably occluded by tyloses. *Rays* 2-4 seriate and up to 40 cells high, heterogeneous with usually 1 but occasionally 2 rows of upright or squarish cells, characterized by the presence of solitary, sporadic, enlarged crystal bearing cells confined to the marginal rows; siliceous inclusions in procumbent cells; horizontal gum canals present characteristically filled with yellow or orange coloured gum; vessel-ray pitting half-bordered, the apertures rounded to irregular in shape; ray cells resin filled. *Parenchyma*, sparse paratracheal. *Fibres* moderately thick walled, septate, characteristically resin plugged in vicinity of septa; pitting inconspicuous, simple.

(iii) *Material*.—*Odina wodier* Roxb., 1 sample India.

Note.—This genus has been included in the survey because of the statement by Pearson and Brown (1932) that one species, namely, *Odina speciosa* Bl., has been recorded from New Guinea. One specimen submitted for determination from this area shows all the characteristics of this genus.

(n) *Parishia**

(i) *General*.—A genus of some 3 or 4 species occurring in Burma and Malaysia. Material examined consisted of 3 unassigned specimens from Malaya and 1 from Borneo. *P. insignis* of Burma is stated to be a large tree up to 130 ft. high and of some local commercial importance for interior finish and case manufacture (Pearson and Brown 1932). Truewood light pinkish-grey darkening to brownish-grey, sapwood wide and often ill-defined from truewood, pale grey-brown. Generally light to moderately light, straight grained or interlocked, texture moderately coarse and even.

(ii) *Structure*.—*Growth rings* not evident. *Vessels* indistinct to the naked eye, moderately numerous and evenly distributed, from 60-75 per cent. solitary, remainder in radial multiples, generally of 2-4; maximum tangential diameter 150-180 μ ; perforation plates simple; inter-vessel pitting moderately fine, alternate, apertures lenticular, borders 8-11 μ ; tyloses not observed. *Rays* 1-2 seriate, low in the Malayan samples but up to 40 cells high in Borneo sample; distinctly heterogeneous with 1 to several rows of upright cells; crystals absent; silica inclusions observed; horizontal gum canals present; vessel-ray pitting half-bordered, the

* Although no definite species were examined the material as a whole was comparable with *Parishia insignis* as described by Pearson and Brown (1932) and therefore the generic description has been included in this survey.

apertures rounded to irregular in shape. *Parenchyma* paratracheal, vasicentric, aliform to confluent; parenchyma cells in transverse section large and prominent. *Fibres* moderately thin to thin walled, non-septate, pits indistinctly bordered.

(iii) *Material*.—*Parishia* spp., 3 samples Malaya, 1 sample Borneo.

(o) *Pentaspadon*

(i) *General*.—A genus of 4 species occurring from Malaya to New Guinea. Medium to large trees, up to 120 ft. high (*P. motleyi*). The New Guinea species of this genus is reported to yield oil when the trunk is tapped (Lane-Poole 1925). The timber in Malaya is not of commercial importance. Truewood pinkish-brown, sapwood pale yellow, somewhat lustrous. Texture moderately fine, straight grained.

(ii) *Structure*.—*Growth rings* not evident. *Vessels* small and indistinct to naked eye, evenly distributed, up to 80 per cent. solitary, remainder in short radial multiples of 2-3; maximum tangential diameters varying from 140-160 μ ; perforation plates simple; inter-vessel pitting moderately fine, alternate, borders 8-9 μ in diameter; tyloses not abundant. *Rays* 2-4 seriate, majority 2-3 seriate, up to 25 cells high; heterogeneous with 1 to several rows of square and upright cells; crystals moderately abundant, chiefly in marginal cells; horizontal gum canals present; vessel-ray pitting half-bordered, the apertures rounded to elongated. *Parenchyma* generally sparse, paratracheal. *Fibres* moderately thin walled, septate; pitting simple and inconspicuous.

(iii) *Material*.—*Pentaspadon minutiflora* B. L. Burt, 1 sample Bougainville I.; *Pentaspadon motleyi* Hook. f., 1 sample New Guinea.

(p) *Pistacia*

(i) *General*.—A small genus of trees or shrubs represented in the south-west Pacific area by 2 tree species; *P. chinensis* Bunge occurs in the Philippine Islands and *P. integerrima* Stewart occurs in mountain forests of north-west India. The trees are small to medium sized. The timbers are not important commercially and are used locally for furniture, carving, and turned articles. Truewood generally olive-brown with narrow dark brown concentric bands. The sapwood is buff coloured sometimes with a yellowish-green tinge, distinct from the truewood. The timber is moderately heavy to heavy, close and fine textured, hard and tough. The pistachio nuts of commerce are produced by *P. vera* L., indigenous to Persia and Syria.

(ii) *Structure*.—*Growth rings* marked by concentric lines of relatively large vessels, ring porous to semi-ring porous. *Vessels* of two sizes, the larger forming a loose, distinct to indistinct ring, maximum tangential diameter from 160-200 μ , generally solitary, occasionally in tangential or radial pairs; the smaller usually in radial multiples of 6-8 or frequently in clusters, 50-60 μ in tangential diameter tending to diminish in size towards the end of the growth ring; perforation plates simple; inter-vessel pitting moderately fine, alternate, borders 9 μ in diameter; spirals present in smaller vessels; tyloses frequent in larger vessels. *Rays* 1-4 seriate, generally 2-3, up to 28 cells high, heterogeneous with 1 or 2 rows of

upright cells, frequently containing large solitary crystals; horizontal gum canals present; vessel-ray pitting half-bordered, the apertures rounded to irregular in shape. *Parenchyma* generally sparse paratracheal, crystals absent. *Fibres* generally non-septate, moderately thick walled to thick walled; pits inconspicuously bordered.

(iii) *Material*.—*Pistacia chinensis* Bunge, 1 sample Philippine Islands.

Note.—Desch (1941) has referred to a Malayan timber placed tentatively in this genus by Mr. C. F. Symington. The timber, however, does not have the ring-porous structure of other members of the genus, nor the horizontal gum canals of *Pistacia chinensis* and *P. integerrima*; in addition, the parenchyma distribution described by Desch does not agree with that of the Indian or Philippine timbers. It would appear doubtful whether the Malayan specimen should be considered under this genus.

(q) *Pleiogynium*

(i) *General*.—A genus consisting of several species of trees occurring in New Guinea, Solomon Islands, and Queensland, Australia. Generally medium sized trees 80-100 ft. high. Truewood reddish-brown to dark red-brown, moderately heavy with fine uniform texture, generally straight grained and fissile.

(ii) *Structure*.—*Growth rings* sometimes indicated by denser bands of fibres. *Vessels* evenly distributed, just visible to the naked eye, moderately numerous (9-12/sq.mm.): 40-50 per cent. solitary, remainder in short radial multiples of 2-3, largest 140-190 μ in tangential diameter; perforation plates exclusively simple, horizontal to oblique; inter-vessel pitting moderately coarse, bordered, alternate, apertures lenticular, horizontal, included; borders generally angular 9-12 μ diameter; tyloses moderately abundant. *Rays* mainly biseriate, up to 25 cells high, heterogeneous with 2-3 rows of upright and squarish cells, with large usually solitary, interspersed crystal bearing cells (Plate 6, Figs. 2 and 3); procumbent cells invariably resin filled; vessel-ray pitting half-bordered, the apertures rounded to irregular in shape; horizontal gum canals present, small and usually resin filled. *Parenchyma* paratracheal, vasicentric to aliform. *Fibres* moderately thick walled, septate fibres sporadic but usually absent, fibres characteristically resin filled, usually in concentric bands or zones; pitting indistinctly bordered.

(iii) *Material*.—*Pleiogynium papuanum* C. T. White, 1 sample Solomon Islands; *P. solandri* Engl., 6 samples Queensland; *Pleiogynium* sp., 1 sample New Guinea.

(r) *Rhodospaera*

(i) *General*.—A genus confined to a single species occurring in New South Wales and Queensland, Australia. *R. rhodanthema* is a medium sized tree reaching a height of 70-80 ft.; the truewood is bright yellow or yellow-brown in colour but is often streaked longitudinally by colour variations, moderately light to moderately heavy, lustrous, straight grained, and fissile. It may be used for decorative cabinet work, but its use is restricted by the small size of log and limited commercial availability.

(ii) *Structure*.—*Growth rings* not distinct. *Vessels* small and indistinct to naked eye, generally in radial multiples of 2-4, evenly distributed, tangential diameter of largest 98μ ; perforation plates simple, horizontal to almost vertical; inter-vessel pitting coarse, bordered, alternate; apertures slit-like, horizontal, borders often angular through crowding, $10-18\mu$ in diameter; tyloses common. *Rays* mainly biseriate, up to 30 cells high, heterogeneous with 1-3 rows of upright cells; crystals abundantly present both in procumbent and upright cells; horizontal gum canals present though infrequent, sometimes sporadic in occurrence, absent in some samples; vessel-ray pitting large, half-bordered, the apertures large, rounded, oval, or irregular in shape; resinous deposits common. *Parenchyma* sparse, paratracheal; crystals absent. *Fibres* sometimes septate, these in some samples confined to region of vessels; pitting fine, inconspicuously bordered.

(iii) *Material*.—*Rhodospaera rhodanthema* Engl., 8 samples Queensland, Australia.

(s) *Rhus*

(i) *General*.—A genus of some 130 species of shrubs and trees distributed throughout subtropical and warm temperate regions. Represented in the south-west Pacific area by several species occurring in Malaya, New Guinea, and the Solomon Islands. Generally small to medium sized trees of the second storey. Truewood usually pale pinkish-brown, streaked and mottled with shades of grey, yellow, and green, rather lustrous; light to moderately light; grain straight or somewhat interlocked, moderately fine textured.

(ii) *Structure*.—*Growth rings* not evident. *Vessels* small and indistinct to the naked eye, evenly distributed, moderately numerous ($6-9/\text{sq. mm.}$) 40-50 per cent. solitary, remainder in radial multiples of 2-3, occasionally up to 5 or 6 in some samples; maximum tangential diameters varying from $150-190\mu$; inter-vessel pitting alternate, apertures lenticular, borders $10-14\mu$ in diameter; perforation plates exclusively simple; tyloses common in *R. simarubaeifolia*. *Rays* generally 2-3 seriate, occasionally uniseriate and up to 4 seriate, up to 25 cells high, heterogeneous with 1 to 2 rows of squarish or upright cells; crystals common both in marginal and procumbent cells; druses fairly common in marginal cells (Plate 5, Fig. 4); resinous deposits moderately abundant to absent; vessel-ray pitting half-bordered, the apertures rounded to irregular in shape; horizontal gum canals not observed in any sample. *Parenchyma* paratracheal to vasicentric; crystals infrequent. *Fibres* thin walled, non-septate, pits inconspicuous, indistinctly bordered.

(iii) *Material*.—*Rhus engleriana* Warb., 1 sample Bougainville I.; *R. simarubaeifolia* A. Gray, 2 samples New Guinea; *R. taitensis* Guillem, 7 samples New Guinea and Solomon Islands.

(t) *Semecarpus*

(i) *General*.—A large genus comprising some 40 species confined to the south-west Pacific area. The trees are medium sized to large, up to 130 ft. high. Some species are characterized by the presence of poisonous sap, similar to that

of species of *Gluta* and *Melanorrhoea* but the virulence varies greatly with the species. Truewood generally pale brown occasionally with pinkish tinge or with pinkish and yellow streaks; light and soft; texture moderately coarse.

(ii) *Structure*.—*Growth rings* indistinct. *Vessels* distinct to just visible to the naked eye, evenly distributed (numbering 2-4/sq.mm.); 40-50 per cent. solitary, remainder in radial multiples of 2-4, occasionally up to 5; maximum tangential diameters varying from 170-270 μ ; perforation plates simple; inter-vessel pitting moderately coarse, apertures lenticular, borders 10-13 μ in diameter; tyloses infrequent. *Rays* generally 2-3 seriate, occasionally up to 4 cells wide, maximum height varying from 20-45 cells high; heterogeneous with 1-4 rows of upright or squarish cells; crystals abundant in marginal and procumbent cells; vessel-ray pitting half-bordered, borders frequently inconspicuous; apertures large, rounded to gash-like; horizontal gum canals absent in all species. *Parenchyma* abundant paratracheal, vasicentric, aliform to frequently confluent; crystal strands occasionally present. *Fibres* generally thin walled, non-septate, pitting inconspicuously bordered.

(iii) *Material*.—*Semecarpus anacardium* Linn. f., 1 sample Bougainville I.; *S. laxiflora* K. Schum., 1 sample Bougainville I.; *S. lucens* King, 1 sample Malaya; *Semecarpus* sp.,* 3 samples New Guinea; *Semecarpus* sp.,* 4 samples New Guinea; *Semecarpus* sp.,* 8 samples New Guinea.

(u) *Spondias*

(i) *General*.—A small genus of trees represented in the south-west Pacific area by 6 species. Several species are widely cultivated for their fruits. Medium to large sized trees up to 110 ft. high. Truewood greyish-brown or yellowish-brown, sapwood ill-defined; light to extremely light, generally straight grained, texture moderately coarse.

(ii) *Structure*.—*Growth rings* sometimes indicated by denser bands of fibres. *Vessels* large and distinct to naked eye, generally few (2-4/sq.mm.), evenly distributed, up to 40 per cent. solitary, remainder of vessels in radial multiples of 2-3, few clusters, maximum tangential diameters up to 330 μ ; perforation plates simple, horizontal; inter-vessel pitting coarse, alternate, the apertures lenticular to slit-like, almost horizontal, borders often angular 12-18 μ in diameter; tyloses thin walled, fairly common. *Rays* generally 4-7 seriate and up to 60 cells high; in some samples a slight tendency to 2 widths, uniseriate, low and infrequent, mostly of upright cells, the remainder 3-7 seriate; heterogeneous with 1-2 rows of upright cells; crystals present in marginal and procumbent cells; horizontal gum canals present, conspicuous with lens on longitudinal faces, microscopically showing a distinct sheath of 1 or 2 cells wide of small epithelial cells; vessel-ray pitting large half-bordered, the apertures rounded or irregular, borders sometimes visible. *Parenchyma* paratracheal, vasicentric to aliform, crystals sometimes present. *Fibres* thin walled, septate, the septa fine and numerous; pits inconspicuous, simple.

* As far as can be told the material from New Guinea forms 3 distinct but unassigned species.

(iii) *Material*.—*Spondias dulcis* Forst., 7 samples New Guinea and Solomon Islands. *S. pinnata* (L.) Kurz, 2 samples New Britain.

(v) *Swintonia*

(i) *General*.—A small genus of trees confined to Burma and Malaysia. Only 1 species, *S. floribunda* Griff., was available for study. This is a large tree 8-9 ft. in girth with a fluted or buttressed base (Pearson and Brown 1932). The true-wood is grey to greyish-brown, moderately light, straight grained, and rather coarse textured; vessel lines prominent; non-durable. In Burma of commercial importance for boat construction and house construction, said to be suitable for match manufacture.

(ii) *Structure*.—*Growth rings* simulated by closely spaced concentric bands of parenchyma. *Vessels* somewhat variable in size, but not graded in the growth ring; the larger solitary, up to 40 per cent. of total, and in radial multiples of 2-3, the smaller frequently in radial multiples up to 6 and in clusters; maximum tangential diameters up to 307 μ ; perforation plates simple; inter-vessel pitting moderately coarse, alternate, apertures lenticular, included, borders 12 μ in diameter. *Rays* mainly biseriate, up to 45 cells high, homogeneous to weakly heterogeneous with one or two rows of squarish cells; crystals absent; siliceous inclusions present; horizontal gum canals in multiseriate rays, resinous deposits moderately abundant; vessel-ray pitting half-bordered, the apertures rounded to irregular in shape, borders usually inconspicuous. *Parenchyma* paratracheal, completely to incompletely vasicentric, but chiefly apotracheal in irregularly spaced continuous to discontinuous bands 2-5 cells wide, also short bands or clumps independent of the pores; crystals not observed. *Fibres* non-septate, moderately thin walled; pits fairly numerous and inconspicuously bordered.

(iii) *Material*.—*Swintonia floribunda* Griff., 1 sample Burma.

IV. FAMILY SUMMARY BASED ON EXAMINATION OF GENERA OCCURRING IN
SOUTH-WEST PACIFIC AREA

(a) *General*

The truewood from the timbers examined is very variable in colour and quite often distinctive and handsome in appearance. Various darker shades of brown or red-brown are found in species of *Dracontomelum*, *Gluta*, *Koordersiodendron*, *Melanochyla*, *Melanorrhoea*, *Odina*, and *Pleiogynium*. Many of the above are streaked with bands of very dark brown to almost black as in *Dracontomelum*. *Rhodosphaera* is a bright yellow to yellow-brown and *Pistacia* is olive to greenish-brown with darker bands. The remainder are generally paler and pinkish to greyish-brown, but frequently streaked with yellow or greenish tints. The lustre of the timbers is variable, usually greatest in the paler coloured, less dense timbers and least in the darker, more dense species. None of the woods examined has any characteristic odour. Densities vary from 25 lb./cu.ft. (air-dry) in *Euroschinus* to 72 lb./cu.ft. (air-dry) in 1 species of *Gluta*. Texture is coarse to moderately fine, and the grain straight or interlocked.

Working properties are variable, many of the genera are characterized by the presence of tension wood, more particularly in the less dense woods, which causes difficulty in sawing and in working with tools generally. Some of the more dense woods, such as *Gluta*, *Pistacia*, and *Melanorrhoea*, take a good finish and are said to be suitable for carving.

(b) Structure

(i) *Growth rings* are, as a whole, not marked, though they are simulated in some genera, namely, *Bouea*, *Gluta*, *Melanorrhoea*, and *Swintonia*, by irregularly spaced bands of parenchyma; *Pistacia* is semi-ring porous showing a loose band of relatively large pores at the commencement of the growth ring, the remainder of the pores being smaller.

(ii) *Vessels* variable in size; large ($290\text{--}330\ \mu$) in *Dracontomelum*, *Gluta*, *Melanorrhoea* (except 1 sp.), *Spondias*, and *Swintonia* to moderately small ($100\text{--}160\ \mu$) in *Campnosperma*, *Euroschinus*, *Mangifera* (*M. indica*), *Pentaspadon*, *Pleiogynium*, and *Rhodosphaera*; distribution even, 60-80 per cent. solitary in *Campnosperma*, *Gluta*, *Mangifera* (1 sp.), *Parishia*, and *Pentaspadon*; remainder solitary and in radial multiples of 2-3 occasionally up to 6; clusters of smaller vessels common in *Pistacia*, infrequent in the rest; those solitary tending to be rounded or sometimes oval with larger axis tangential: perforation plates exclusively simple, horizontal to inclined, except in *Campnosperma*, where scalariform and simple plates occur together, the scalariform sometimes predominating, with fine bars up to 20 in number; a reticulate type of perforation plate (Plate 6, Fig. 1) quite common in one or two samples of *Euroschinus*, together with the predominating simple plate: inter-vessel pitting bordered, alternate, apertures lenticular to slit-like, included and generally horizontal to sometimes obliquely inclined; borders rounded or angular due to crowding, diameters varying from medium sized ($7\text{--}14\ \mu$) in the majority of genera, to large and conspicuous (up to $18\ \mu$ in *Dracontomelum*, *Rhodosphaera*, and *Spondias*: tyloses abundant in some genera, notably *Dracontomelum*, *Gluta*, *Koordersiodendron*, *Melanorrhoea*, *Microstemon*, *Odina*, *Pentaspadon*, *Pleiogynium*, and *Rhodosphaera*: spirals absent except in smaller pores of *Pistacia*.

(iii) *Rays* varying from 4-7 cells wide in *Spondias* and occasionally in *Dracontomelum* to uniseriate or partly biseriate in *Bouea*, *Gluta*, *Melanorrhoea*, *Mangifera* (1 sp.), and *Parishia*, the remainder generally 2-3 seriate, occasionally up to 4 seriate; typically heterogeneous in majority of genera with 1-4 rows of upright or squarish cells; homogeneous or nearly so in *Gluta* and *Melanorrhoea*; crystals abundant, typically in square or upright marginal cells, usually solitary; characteristically enlarged solitary crystal bearing cells occur sporadically in the marginal cells of *Odina*, *Pistacia*, and *Pleiogynium* (Plate 6, Figs. 2 and 3); druses observed in upright and procumbent cells of 2 spp. of *Rhus* (Plate 5, Fig. 4); deposits of siliceous material observed in rays of *Gluta*, *Melanorrhoea*, *Odina*, *Parishia*, and *Swintonia*: ray height variable from a maximum of 15 cells in *Gluta* to 60 cells in *Spondias*, majority between 25 and 40 cells high: vessel-ray pitting

generally coarse, half-bordered, the borders frequently indistinct and corresponding to the aperture, rounded, irregular to elongated vertically or horizontally, tending to be scalariform in *Campnosperma*, *Euroschinus*, *Rhus*, and *Semecarpus*; radial gum canals, variable in size between genera, observed in all genera except *Blepharocarya*, *Bouea*, *Dracontomelum*, *Mangifera*, *Rhus*, and *Semecarpus*, sometimes large and conspicuous with definite epithelial lining, as in *Spondias*, but usually smaller, sometimes with clear yellow gum deposits: resinous deposits abundant to absent.

(iv) *Parenchyma* paratracheal, vasicentric to greater or lesser degree in all genera except *Campnosperma* where parenchyma is absent or extremely sparse paratracheal; aliform to confluent in *Dracontomelum*, *Mangifera*, *Melanochyla*, *Parishia*, *Pleiogynium*, *Semecarpus*, and *Spondias*; apotracheal in irregularly spaced bands, narrow to moderately wide continuous or short and discontinuous in *Bouea*, *Gluta*, *Melanorrhoea*, and *Swintonia*; similar bands occur infrequently in *Mangifera*, though the majority of bands are of paratracheal origin: crystal strands sparse in *Dracontomelum*, *Euroschinus*, *Koordersiodendron*, *Pleiogynium*, *Semecarpus* (2 spp.), and *Spondias*; resinous deposits usually present.

(v) *Fibres* thick walled to thin walled, regularly septate in *Dracontomelum*, *Koordersiodendron*, *Microstemon*, *Odina*, *Pentaspadon*, and *Spondias*, sparsely septate in *Blepharocarya*, *Campnosperma*, *Euroschinus*, *Pleiogynium*, and *Rhodosphaera*, non-septate in the remainder; pitting generally inconspicuous, simple or indistinctly bordered; sometimes resin filled.

(vi) *Tracheids* absent.

(vii) *Ripple marks* not observed.

V. DISCUSSION

In reviewing the anatomy of the family as revealed in this survey it can be stated that:

- (i) All genera have large, half-bordered (the borders often inconspicuous), rounded to irregularly shaped vessel-ray pits which are sometimes in scalariform arrangement.
- (ii) Sixteen of the genera examined have horizontal gum canals in the rays.
- (iii) Crystals, either in the marginal ray cells or to a lesser extent in the procumbent cells, are a characteristic feature of 16 genera; 5 genera show numerous deposits of siliceous material in the rays.
- (iv) Septate fibres occur regularly in 6 genera and are sporadic in occurrence in 5 genera.
- (v) The parenchyma is of the paratracheal type, although some apotracheal occurs in some genera.

The best basis for separating or grouping the various genera appears to be on the occurrence and abundance of the parenchyma. Therefore, three main

divisions have been made as follows:

- A. Parenchyma paratracheal but also in apotracheal bands.
- B. Parenchyma paratracheal, aliform to confluent.
- C. Parenchyma sparse paratracheal, or paratracheal to vasicentric.

It will be realized that in such a grouping there will be a possibility of overlap between divisions B and C since vasicentric parenchyma merges with aliform.

TABLE 2

GROUPING OF 22 GENERA OF THE ANACARDIACEAE OCCURRING IN THE SOUTH-WEST PACIFIC AREA ON (1) BOTANICAL GROUNDS AND (2) ANATOMICAL GROUNDS

(1) Botanical grouping by Engler taken from Willis (1931)	(2) Wood anatomical grouping as suggested in this paper.
A. Five free carpels or 1, leaves simple, entire	A.—Parenchyma paratracheal and in apotracheal bands
1. Mangiferae	
<i>Bouea</i>	<i>Bouea</i>
<i>Buchanania</i>	
<i>Gluta</i>	<i>Gluta</i>
<i>Mangifera</i>	<i>Mangifera</i>
<i>Melanorrhoea</i>	<i>Melanorrhoea</i>
<i>Swintonia</i>	<i>Swintonia</i>
B. Carpels united, leaves rarely simple	B.—Parenchyma paratracheal, aliform to confluent
2. Spondiac	
<i>Dracontomelum</i>	<i>Buchanania</i>
<i>Koordersiodendron</i>	<i>Dracontomelum</i>
	<i>Koordersiodendron</i>
	<i>Melanochyla</i>
<i>Odina</i>	
<i>Pleiogynium</i>	<i>Pleiogynium</i>
	<i>Semecarpus</i>
<i>Spondias</i>	<i>Spondias</i>
3. Rhoideae	C.—Parenchyma sparse paratracheal, or paratracheal to vasicentric
<i>Blepharocarya</i>	<i>Blepharocarya</i>
<i>Camptosperma</i>	<i>Camptosperma</i>
<i>Euroschinus</i>	<i>Euroschinus</i>
<i>Microstemon</i>	<i>Microstemon</i>
	<i>Odina</i>
<i>Parishia</i>	<i>Parishia</i>
<i>Pentaspadon</i>	<i>Pentaspadon</i>
<i>Pistacia</i>	<i>Pistacia</i>
<i>Rhodosphaera</i>	<i>Rhodosphaera</i>
<i>Rhus</i>	<i>Rhus</i>
4. Semecarpeae	
<i>Melanochyla</i>	
<i>Semecarpus</i>	

However, the genera do seem to fall naturally into three groups and on this basis there is very close agreement with the division of the family on botanical grounds (see Table 2). The most obvious exception is *Buchanania* which, on the basis of wood anatomy, has been placed in Section B but which botanically is in the Tribe Mangiferae (corresponding to Section A anatomically). *Melanochyla* and *Semecarpus* are in the Tribe Semecarpeae, which has no counterpart in the grouping on anatomical grounds. They have been placed in Section B on the basis of their anatomy.

The genus *Campnosperma* is somewhat anomalous anatomically, as it possesses very sparse parenchyma and scalariform perforation plates. It is the only genus in the family that has distinct scalariform perforation plates. The genus *Mangifera* appears to form a link between Sections A and B in that it shows apotracheal parenchyma but also has the paratracheal to confluent parenchyma well developed.

The genera in each section can be subdivided on the presence or absence of septate fibres and on the presence or absence of horizontal gum canals in the rays.

It is not always an easy matter to place a timber in its correct family on the basis of anatomical features alone. Many families do have characteristics which serve to place them positively, but in other cases woods of several families possess characteristics in common.

The woods of the Burseraceae occurring in the area under consideration have certain characteristics in common with some of those of the Anacardiaceae; these are: septate fibres; horizontal gum canals in the rays; coarse, alternate inter-vessel pitting; simple perforation plates; coarse half-bordered, or apparently simple to scalariform vessel-ray pitting; paratracheal parenchyma.

The timbers of the Burseraceae are characterized by a paucity of parenchyma which is generally sparsely paratracheal or incompletely vasicentric; thus confusion may arise between this family and certain genera of Section C, above, of the Anacardiaceae. Of the genera in this section *Campnosperma* can be readily recognized by the presence of scalariform as well as simple perforation plates, and *Pistacia* shows a ring porous structure absent from all genera of the Burseraceae. Septate fibres are of regular occurrence in the Burseraceae, and of the remaining genera in Section C septate fibres are sparse to absent in *Blepharocarya*, *Euroschinus*, *Parishia*, *Rhodosphaera*, and *Rhus*; thus confusion might arise only with *Microstemon*, *Odina*, and *Pentaspadon*.

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VII. REFERENCES

- BOAS, I. H. (1947).—"The Commercial Timbers of Australia, their Properties and Uses." Coun. Sci. Industr. Res. Aust. Publ. (Govt. Printer: Melbourne.)

- BURKILL, I. H. (1935).—"A Dictionary of the Economic Products of the Malay Peninsula." 2 Vols. Published for the Governments of the Straits Settlements and Federated Malay States by the Crown Agents for the Colonies, London.
- COCKRELL, R. A. (1934. Revised 1941).—"An anatomical study of eighty Sumatran woods. A dissertation submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in the University of Michigan.
- DESCH, H. E. (1941).—"Manual of Malayan Timbers." Vol. 1. Malayan Forest Records No. 15. (Caxton Press Ltd.: Kuala Lumpur.)
- FRANCIS, W. D. (1929).—"Australian Rain Forest Trees." (Govt. Printer: Brisbane.)
- HESS, R. W. (1946).—"Identification of New World timbers. Part II. Anacardiaceae. *Trop. Woods* No. 87: 11.
- JANSSONIUS, H. H. (1908).—"Mikrographie des Holzes der auf Java Vorkommenden baumarten." Vol. 2. (E. J. Brill: Leiden.)
- KEITH, H. G. (1938).—"A Preliminary List of North Borneo Plant Names." North Borneo Forest Records No. 2. (Government Printing Office: Sandakan, North Borneo.)
- KEITH, H. G. (1947).—"The Timbers of North Borneo." North Borneo Forest Records No. 3. (Ye Olde Printerie: Hong Kong.)
- LANE-POOLE, C. E. (1925).—"The Forest Resources of the Territories of Papua and New Guinea." Parliamentary Rep. (Govt. Printer: Melbourne.)
- MEAD, J. P. (1928).—"The Forests of the Colony of Fiji." Council Paper No. 4, Legislative Council, Fiji. (Govt. Printer: Suva.)
- MERRILL, E. D. (1946).—"Plant Life of the Pacific World." (The Macmillan Co.: New York.)
- PEARSON, R. S., and BROWN, H. P. (1932).—"Commercial Timbers of India." (Govt. of India, Central Publications Branch.)
- RECORD, MARY (1945).—"A collection of woody plants from Melanesia. *Trop. Woods*. No. 81: 9.
- RECORD, S. J., and HESS, R. W. (1943).—"Timbers of the New World." (Yale Univ. Press: New Haven.)
- REYES, L. J. (1938).—"Philippine Woods. Techn. Bull. No. 7. (Bureau of Printing; Manila.)
- SWAIN, E. H. F. (1928).—"The Timbers and Forest Products of Queensland." (Govt. Printer: Brisbane.)
- WILLIS, J. C. (1931).—"A Dictionary of the Flowering Plants and Ferns." (Cambridge Univ. Press: Cambridge.)

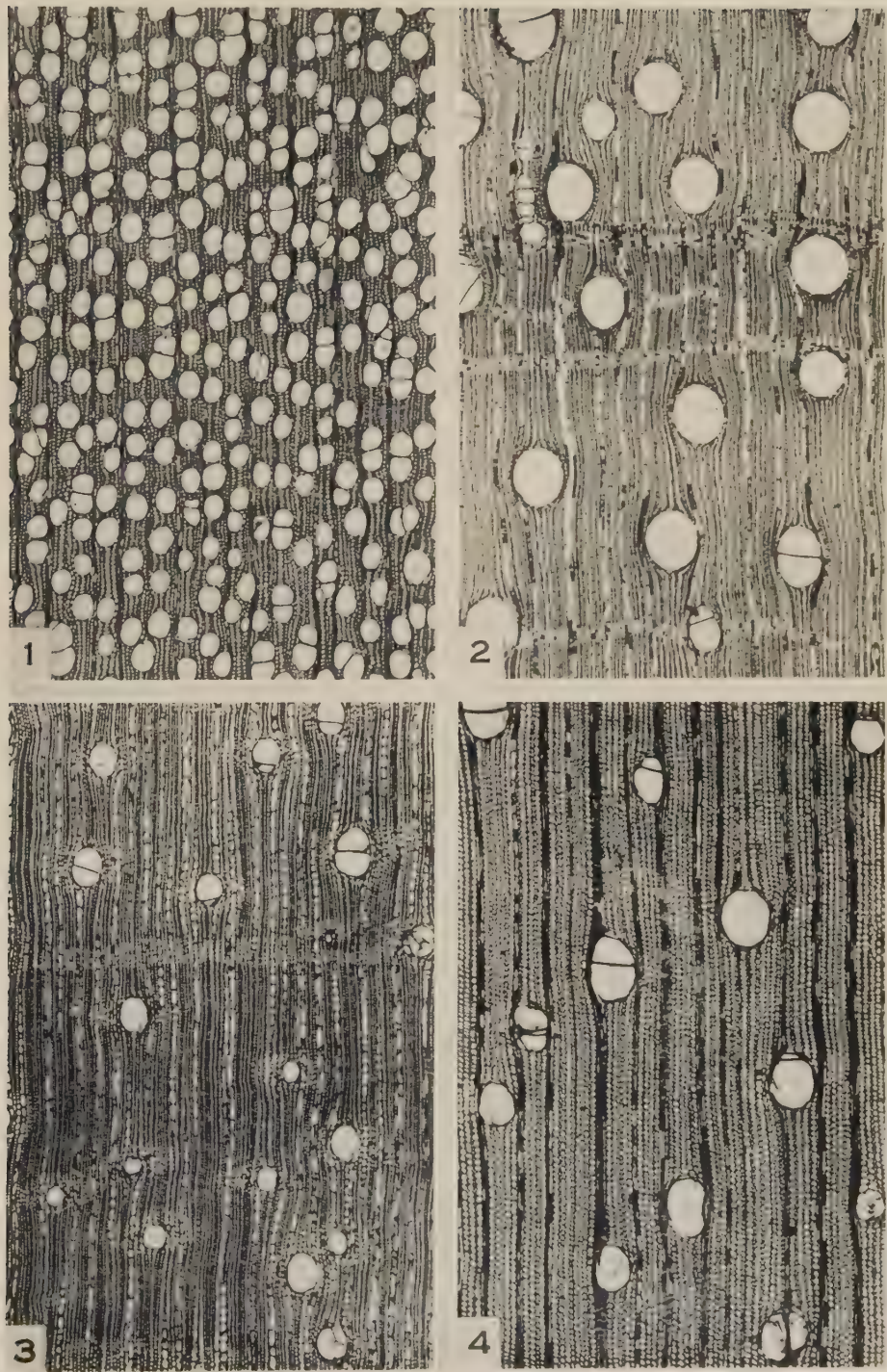
EXPLANATION OF PLATES 1-6

PLATE 1

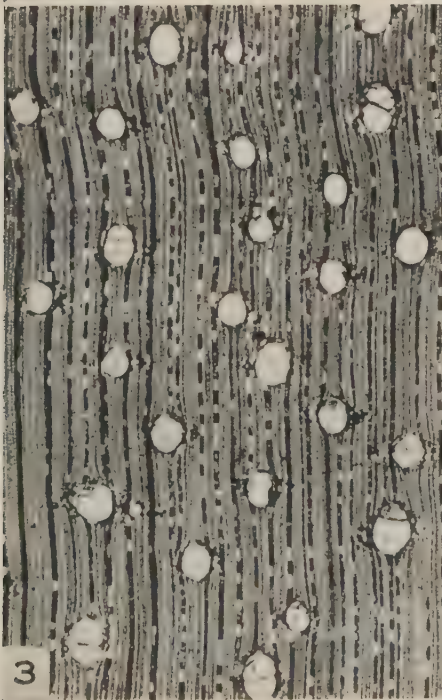
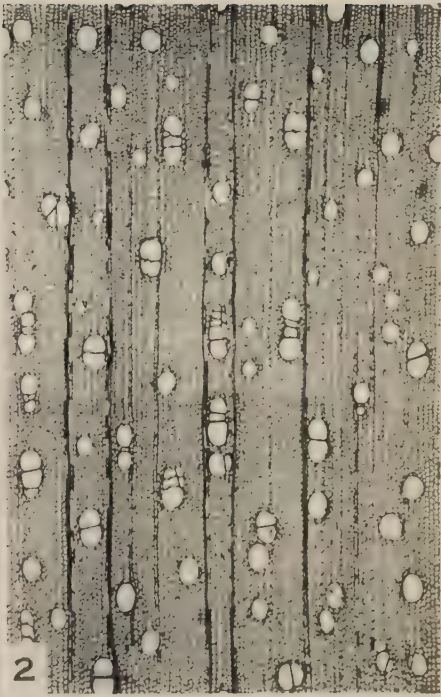
- Fig. 1.—*Campnosperma brevipetiolata* Volkens. Transverse section showing size and arrangement of pores and absence of parenchyma. x 25.
- Fig. 2.—*Swintonia floribunda* Griff. Transverse section showing large pores and apotracheal bands of parenchyma. x 25.
- Fig. 3.—*Mangifera minor* Bl. Transverse section showing vasicentric, aliform parenchyma, and portion of apotracheal band. x 25.
- Fig. 4.—*Semecarpus* sp. Transverse section showing resin filled rays and aliform parenchyma. x 25.

PLATE 2

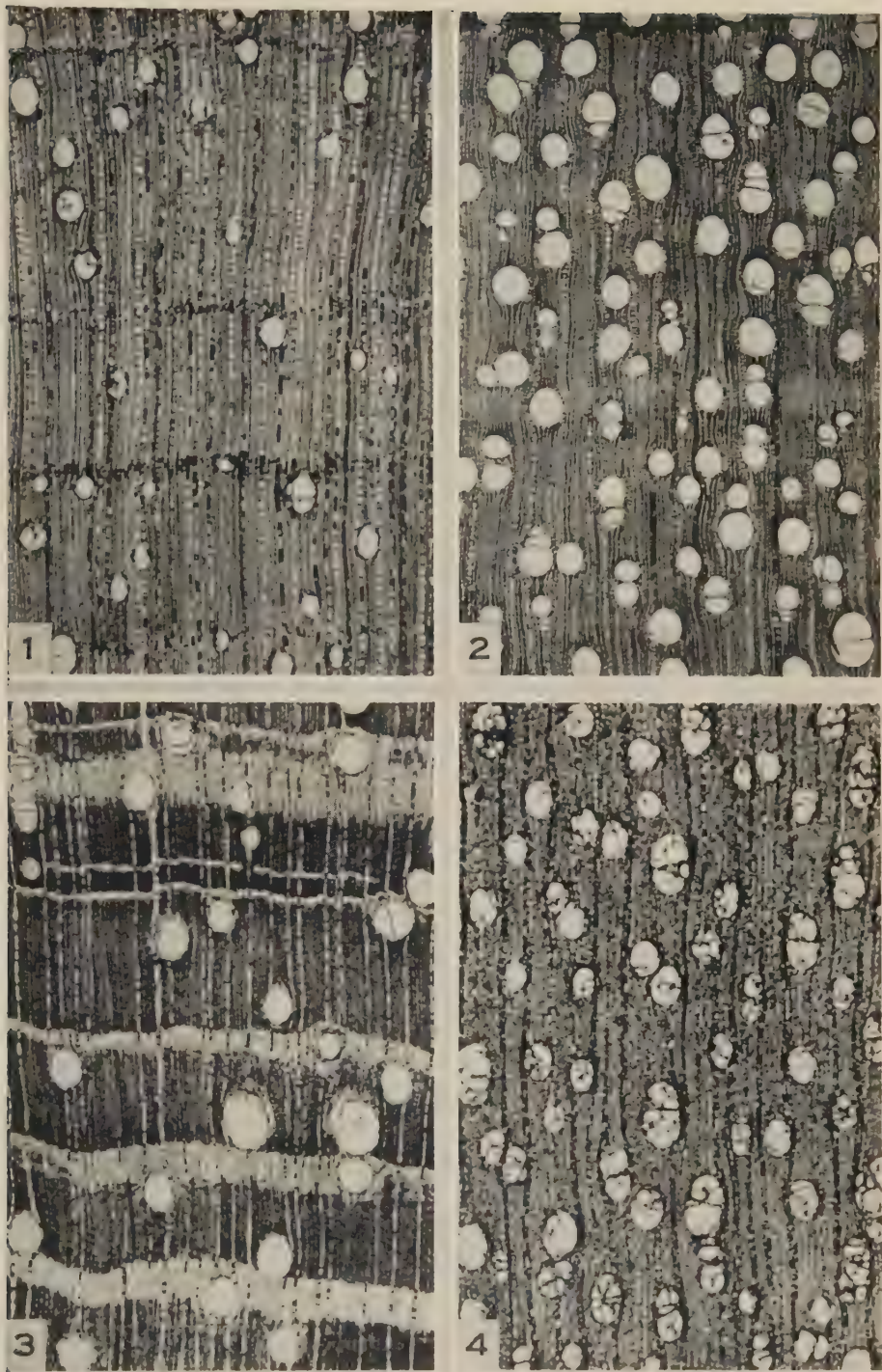
- Fig. 1.—*Pistacia chinensis* Bunge. Transverse section showing semi-ring porous structure and characteristic grouping of smaller pores. x 25.
- Fig. 2.—*Euroschinus falcatus* Hook. Transverse section showing size and arrangement of pores and thin walled fibres. x 25.
- Fig. 3.—*Melanochyla kunstleri* King. Transverse section showing vasicentric, aliform parenchyma, and typical appearance of resin in rays. x 25.
- Fig. 4.—*Buchanania lucida* Bl. Transverse section showing occasional clustered arrangement of pores. x 25.



DADSWELL and INGLE.—THE ANATOMY OF THE TIMBERS OF THE SOUTH-WEST PACIFIC AREA.
I. ANACARDIACEAE



DADSWELL and INGLE.—THE ANATOMY OF THE TIMBERS OF THE SOUTH-WEST PACIFIC AREA.
I. ANACARDIACEAE

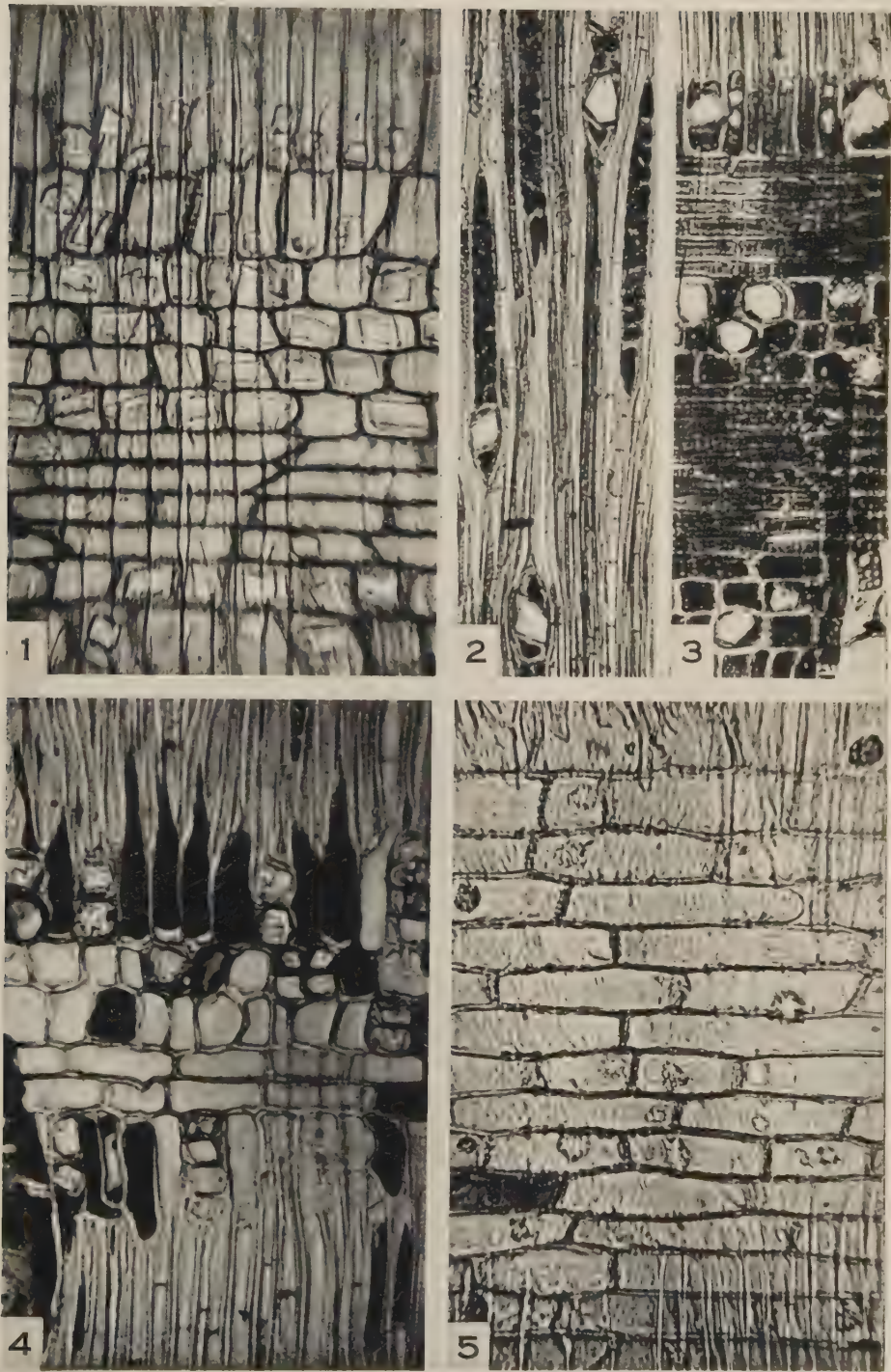


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DADSWELL and INGLE.—THE ANATOMY OF THE TIMBERS OF THE SOUTH-WEST PACIFIC AREA.
I. ANACARDIACEAE

PLATE 3

- Fig. 1.—*Bouea macrophylla* Griff. Transverse section showing numerous uniseriate rays and resin-filled bands of apotracheal parenchyma. $\times 25$.
Fig. 2.—*Microstemon velutina* Engl. Transverse section showing pore arrangement and sparse paratracheal parenchyma. $\times 25$.
Fig. 3.—*Melanorrhoea laccifera* Pierre. Transverse section showing large pores, frequently solitary, apotracheal bands of parenchyma and wide bands of resin-filled fibres. $\times 25$.
Fig. 4.—*Odina wodier* Roxb. Transverse section showing pores characteristically tylosed. $\times 25$

PLATE 4

- Fig. 1.—*Euroschinus falcatus* Hook. Radial section showing reticulate perforation plate. $\times 400$.
Fig. 2.—*Campnosperma minor* Corner. Scalariform perforation plate exposed on split radial surface of wood. $\times 220$.
Fig. 3.—*Dracontomelum mangiferum* Bl. Tangential section of vessel showing coarse bordered pitting (average size of pits 18μ). $\times 220$.
Fig. 4.—*Blepharocarya involucrigera* F.v.M. Tangential section of vessel showing fine bordered pitting (average size of pits 8μ). $\times 220$.

PLATE 5

- Fig. 1.—*Microstemon velutina* Engl. Tangential section showing small horizontal gum canal in ray and septate fibres. Note upright cells in rays marginal and interspersed. $\times 150$.
Fig. 2.—*Spondias dulcis* Forst. Tangential section showing large horizontal gum canals in rays. $\times 90$.
Fig. 3.—*Semecarpus lucens* King. Radial section showing typical vessel-ray pitting found in the Anacardiaceae. $\times 200$.
Fig. 4.—*Rhus taitensis* Guillem. Radial section showing crystal druses in ray cells. $\times 750$.

PLATE 6

- Fig. 1.—*Rhus taitensis* Guillem. Radial section showing location of crystals in rays typical of many of the genera of Anacardiaceae. $\times 260$.
Figs. 2 and 3.—*Pleogynium solandri* Engl. Tangential and radial sections showing enlarged crystal bearing cells in the rays. $\times 150$.
Fig. 4.—*Koordersiodendron pinnatum* (Blanco) Merr. Radial section showing scattered arrangement of crystal bearing cells and elongated marginal upright ray cells characteristic of this genus. $\times 220$.
Fig. 5.—*Gluta reinghas* L. Radial section showing homogeneous ray and silica deposits in the ray cells. $\times 260$.

SOME FACTORS AFFECTING LOCALIZED AND SYSTEMIC NECROTIC REACTIONS TO VIRUS Y IN THE POTATO

By E. M. HUTTON*

(Plates 1-2)

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Summary

Factors influencing hypersensitive and necrotic reactions to virus Y in potato seedlings, developed from common varieties like Katahdin and Snowflake, have been studied mainly by means of hand inoculation in the greenhouse. This method provides more accurate and detailed information than either graft inoculation or aphid inoculation. The virus dosages applied by aphid inoculation have not been sufficient to assess the hypersensitivity of seedlings accurately.

The strain of virus Y used in inoculation is an important factor determining whether a seedling will give a localized reaction or not. A seedling hypersensitive to one strain has a one in two chance of being hypersensitive to a different strain. Seedlings hypersensitive to all strains of Y tested have been developed. Strains of virus Y differed in the virus concentrations which they developed in tobacco, but this did not appear to be a factor influencing the relationship between strain of Y and the hypersensitive reaction of seedlings.

In a number of the seedlings, lethal and localized necrotic reactions were interchangeable, depending on plant vigour at the time of inoculation. Young actively growing plants of hypersensitive seedlings give reactions localized in the inoculated leaves, while old plants or plants with an apparently lowered metabolism give a diffused type of necrotic reaction which results in the inactivation of the virus in tissues distant from the inoculation sites. This is possible, because the stem tissue of such seedlings can react hypersensitively to virus Y. Histological studies confirmed these findings, and showed a correlation between the extent of necrosis in stem sections and the degree of sensitivity to virus Y.

I. INTRODUCTION

Immunity in potatoes to a potato virus was obtained for the first time with the American seedling U.S.D.A. 41956 (Schultz *et al.* 1934) which is immune to all strains of virus X, and which transmits this factor to hybrid progeny (Stevenson, Schultz, and Clark 1939). Up to the present, immunity or non-susceptibility to the other potato viruses has not been found. It was discovered, however, that some varieties and their seedlings were resistant to the mosaic viruses A and X (Cockerham 1943) under field conditions due to the extreme susceptibility of their tissues. This hypersensitivity, which is heritable (Cadman 1942), prevents the spread of the virus because of the rapidity of tissue death around the infection points of inoculated leaves. Consequent upon these findings, the elimination of viruses A and X from potato stocks has been made possible by the development of new varieties possessing these genetic factors.

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The development of field immunity to virus Y, the other important mosaic disease, has proved a difficult problem. Necrotic reactions to this virus, resulting in high field resistance, had been observed in the potato variety Katahdin and its progeny in the United States (Jones and Vincent 1937; Jones, Vincent, and Burk 1940), and in Snowflake in Australia (Bald and Pugsley 1941). Work in progress at Canberra (Hutton and Bald 1945; Hutton 1945, 1946) has had as its objective the development of seedlings hypersensitive to virus Y, using as basic parental material the necrotic reacting Katahdin and Snowflake varieties. The reactions of the seedlings to hand inoculation with virus Y in the greenhouse have been described, as well as the inheritance of the various types of necrotic reaction obtained (Hutton 1945, 1946).

Cockerham (1945) and Cockerham and M'Ghee (1947) found clones of three wild *Solanum* species with hypersensitive reactions to virus Y, and in tests with progenies from two of them, *S. demissum* and *S. simplicifolium*, observed the reactions to be heritable and to result in immunity to virus Y under field conditions. It is apparent from this work and the research on virus Y resistance previously quoted (Hutton 1945, 1946) that varieties field immune to this virus are in sight.

Recently, Bawden and Kassanis (1946), Roland (1946), and Schultz, Stevenson, and Akeley (1947) have examined the bases for differences in susceptibility to virus Y found among common potato varieties. Bawden and Kassanis (1946) found that a variety like Majestic, reacting with leaf-drop streak, developed a lower concentration of virus Y and was as resistant as Arran Banner, which reacted with a mosaic and developed a high concentration of this virus. They found the reaction of Katahdin to virus Y to approximate hypersensitivity. Roland (1946) concluded that defective diffusion of virus Y rather than sensitivity was the reason for the tuber progeny of infected plants being free from this virus. Schultz, Stevenson, and Akeley (1947) found a severe necrotic reaction and field resistance to virus Y to be correlated, and emphasized the effect that a heavy aphid infestation giving the chance for repeated plant inoculation has on the degree of infection in varieties.

The work described in this paper was done with the object of finding what factors, apart from genetic, influenced or modified the expression of the localized and other necrotic reactions in the potato seedlings developed at Canberra. These seedlings have all been selected from crossbred progenies involving common potato varieties as parents, so that it is possible that a different gene complex from that present in Cockerham and M'Ghee's (1947) hybrids with wild species is operating to produce the necrotic reactions obtained. The Canberra progenies contained seedlings with individual reactions to virus Y ranging through complete tolerance, leaf-drop streak, and severe systemic necrosis to hypersensitivity. Greenhouse tests over several years have shown a number of the localized reactors to be more sensitive than Katahdin under most conditions, whereas a proportion of them appear to have inherited a similar reaction to Katahdin. Limited field tests have largely supported the greenhouse tests, but it is apparent that the level of necrotic reaction required for field immunity is dependent on the density of

the aphid population present. With relatively low aphid populations, the Katahdin type of reaction results in near field immunity under Australian conditions, and it is only when seasonal conditions optimal for aphid development are obtained, that a noticeable infection occurs in Katahdin and seedlings with a similar reaction to virus Y.

II. MATERIALS AND METHODS

In this study, the 342 seedlings used were those selected in the 1945-46 season as having the best agronomic characters in the Dickson Experiment Station field plots containing hybrid progenies bred for virus Y resistance. As they were selected initially on an agronomic basis, they included types giving localized, lethal necrotic, systemic necrotic, and mottle reactions to virus Y in the greenhouse. With respect to the latter two classifications, a systemic necrotic seedling usually showed some mottling, and a seedling classed as a mottle type often had some systemic necrosis. For convenience, seedlings with leaf-drop streak were classified as having systemic necrosis.

In the preliminary stages of this study all the field selected seedlings were involved, but during the later stages of the work on the effect of strain on resistance, 50, and then 13, seedlings were selected. The varieties Factor and Katahdin were used as checks throughout, and in some instances Sequoia was used as well.

Most of the greenhouse inoculations were done during the spring and summer period from October to March when growth of potatoes is active and relatively normal. For comparison, however, some inoculations were made at other times of the year when vegetative growth was restricted due to the shorter photoperiod and lowered temperatures (Werner 1934). The varieties and seedlings were hand inoculated at various stages of growth with a ground glass spatula after the leaves to be inoculated were tagged and dusted lightly with an "extra fine" grade of carborundum. The inoculum was prepared in a mortar by grinding virus Y infected tobacco leaves (showing vein banding) with a phosphate buffer at pH7 in the constant proportion of 1:20 (w/v). Aphid inoculations were done with *Myzus persicae*, using the procedure of Watson and Roberts (1939).

For the 1945-46 and 1946-47 seasons the strain of virus Y used was that found in the Carman potato variety in the Ballarat district of Victoria. In the 1947-48 season, the work on the effect of the strain of virus Y on hypersensitivity was made possible through the generosity of F. C. Bawden who supplied the cultures of a highly virulent strain, Rothamsted, a strain of medium virulence known as Sheffield, and a mild strain designated 18.2 from the Rothamsted Experimental Station, England. Virus C had been obtained previously from Dr. J. G. Bald (Bald and Norris 1945), and this had been kept in Brown's River free from all other viruses. As a result, comparisons of the effects of Victorian Y, the three Rothamsted strains of Y, and virus C were made on selected seedlings.

When not being used for inoculation, the strains of virus Y, with the exception of C, were kept in the variety Epicure which, due to its hypersensitive reactions, remains free from viruses A, C, and X. For inoculation purposes, the virus Y strains including C (Bawden and Sheffield 1944) were transferred by hand

inoculation to young tobacco plants. In order to maintain the strains in high concentration they were transferred each month to a fresh batch of young tobacco plants.

III. CORRESPONDENCE BETWEEN THE RESULTS OBTAINED WITH THE SAME SEEDLINGS IN TWO DIFFERENT SEASONS

Duplicate samples of the 342 field selected seedlings were hand inoculated in the greenhouse with Victorian Y in both the 1945-46 and 1946-47 seasons. In the 1945-46 season, the seedlings were inoculated before flowering in November, while in the 1946-47 season this inoculation was done at approximately the same time, but those with localized reactions were given a second inoculation after flowering. In both seasons the tubers from the localized reactors were kept, and when these were grown on, the plants were healthy and gave typical localized necrotic reactions on reinoculation with virus Y.

A fair correspondence between the results from the seedlings in two different seasons at the pre-flowering inoculation is shown in Table 1. Of the localized reactors in the 1945-46 season, 72 per cent. gave the same reaction in the 1946-47 season, the rest developing systemic necrosis. Twelve of this latter group were early types which did not flower, and were inoculated at the equivalent of their post-flowering stage in the 1945-46 season, so that their quick maturation had localized the virus. In the 1946-47 season, all seedlings were inoculated at what was judged to be their pre-flowering growth stage, so that rapid maturation of early types was allowed for, and the results generally were thus more accurate.

TABLE 1

TWOFOLD FREQUENCY DISTRIBUTION OF 342 POTATO SEEDLINGS, CLASSIFIED ON PLANT REACTION AFTER HAND INOCULATION AT THE PRE-FLOWERING STAGE WITH VICTORIAN Y IN THE 1945-46 AND 1946-47 SEASONS

Plant Reactions 1945-46 Season	Plant Reactions 1946-47 Season				Total
	Localized	Lethal Necrosis	Systemic Necrosis	Mottle	
Localized	49	0	19	0	68
Lethal necrosis	40	11	90	0	141
Systemic necrosis	0	0	35	10	45
Mottle	0	0	33	55	88

In Table 1, of the seedlings which were lethally necrotic to Victorian Y in the 1945-46 season, 8 per cent. gave the same reaction in the 1946-47 season and 28 per cent. localized this virus, the rest developing systemic necrosis after inoculation. These results indicated that lethal and localized necrosis can be interchangeable in the same seedling, and that the separation of true lethal from systemic necrotic seedlings is influenced by some factor like plant vigour. In the 1945-46 season, the plants came from small tubers raised in pots in the greenhouse from first year seedlings, and were on the average less vigorous than the 1946-47 duplicates from field grown tubers.

Correspondence between systemic necrotic and mottle types in the two seasons was close, when due allowance is made for the fact that the one reaction is usually accompanied by the other to a greater or lesser extent.

(a) *The Effect of Plant Vigour on the Reaction to Virus Y in Potato Seedlings*

In order to investigate whether plant vigour could influence the final reaction to virus Y, vigour ratings recorded in the 1944-45 season were examined, and the results for the 1,074 necrotic reacting seedlings analysed. The vigour rating was made at inoculation, which was carried out at the pre-flowering stage. Those seedlings which were weak and spindly at inoculation were given a rating of 1, those which were strong growing and vigorous, a rating of 5, and the rest appropriate ratings intermediate between these two categories.

Table 2 shows a decided fall in the number of lethal necrotic seedlings, and less marked rises in the numbers of systemic necrotic and localized reactors to virus Y, passing from the least vigorous to the most vigorous plant classes. These results make it clear that plant vigour can markedly influence the virus Y reaction in some seedlings. It has been observed repeatedly, that if weakly growing

TABLE 2

PERCENTAGES OF SEEDLINGS OF DIFFERENT VIGOUR RATINGS AT INOCULATION IN RELATION TO THEIR FINAL Y REACTION CLASSES

Vigour Rating	Percentage of Seedlings in the Different Reaction Classes			Number of Seedlings
	Systemic Necrosis	Lethal Necrosis	Localized Necrosis	
1	58.1	36.0	5.9	86
2	60.4	35.2	4.4	270
3	69.2	24.2	6.6	335
4	77.0	16.1	6.9	204
5	84.3	6.7	9.0	179

seedlings develop lethal necrosis after inoculation, this may not be their normal reaction, as strongly growing duplicate material will give either a localized or systemic necrotic reaction. This explains the inconsistencies of Table 1 with the lethal necrotic class, since the 1946-47 material was on the average much more vigorous than that grown the previous season. The results in Table 2 indicate that to obtain the most reproducible results from hand inoculation with virus Y in the greenhouse, it is necessary to plant tubers which will give vigorous plants.

IV. THE RELATION BETWEEN STAGE OF GROWTH OF SEEDLINGS AT INOCULATION AND LOCALIZED REACTION TO VIRUS Y

The 89 seedlings shown in Table 1, as giving in the 1946-47 season a localized reaction to virus Y when inoculated at the pre-flowering stage, were given a second inoculation 14 days after the commencement of flowering. At this stage a large proportion of the seedlings had completed flowering, although some of the later types were still producing a few flowers.

The results in Table 3 show that a number of the seedlings with localized reactions after the pre-flowering inoculation developed systemic necrosis following the post-flowering inoculation. In a few of the seedlings, the systemic necrosis was severe and almost lethal in the maturing plants, whereas in the remainder it was light, being evident as a few small veinal lesions on 3 to 6 leaflets in different positions on the plant. The Factor checks developed leaf-drop streak after the first inoculation, while the Katahdin checks localized the virus, but developed systemic necrosis after the second inoculation.

TABLE 3

THE EFFECT OF STAGE OF GROWTH AT INOCULATION ON THE SUBSEQUENT REACTION OF 89 POTATO SEEDLINGS TO VIRUS Y

Number Localized After 1st Pre-flowering Inoculation	Result of 2nd Post-flowering Inoculation	
	Number Localized	Number of Systemic Necrosis
89	44	45

It appeared from the results in Table 3, that the physiological plant response which causes localization of virus Y, was not rapid enough in half of the seedlings at the second inoculation to prevent systemic invasion of tissues away from the inoculation sites. In contrast, the tendency in very early type seedlings, as mentioned previously, is for the virus to be localized owing to the rapid maturation.

The results of Table 3 made it necessary to find to what extent the hypersensitive reaction had failed to prevent systemic infection in the seedlings in which localization was incomplete at the second inoculation. Further, it was of importance to know whether the systemic necrosis resulting after the post-flowering inoculation was of the same type as that previously obtained after the pre-flowering inoculation in many seedlings. In order to investigate these points, tubers from some of the seedlings of both groups in Table 3 were kept and planted in May 1947.

TABLE 4

EMERGENCE RESULTS FROM THE TUBERS OF SEEDLINGS INOCULATED PREVIOUSLY WITH VICTORIAN Y AT PRE- AND POST-FLOWERING GROWTH STAGES

Results of Hand Inoculation November and December 1946		Symptoms in Plants from 2 Tubers of Each Seedling			Total Seedlings
Pre-flowering	Post-flowering	Both free of Y	One Plant free of Y, one with Necrosis	Both Plants with Necrosis	
Localized	Localized	12	0	0	12
Localized	Systemic necrosis	8	3* + 1	4* + 4	20
Localized	Lethal necrosis	1	0	1*	2

Note.—Seedlings marked with an asterisk produced one or two plants with lethal necrosis. The other plants developed a severe necrosis which was almost lethal. Freedom from virus Y was proved by inoculation to 11-84 a hypersensitive seedling used as an indicator plant.

Table 4 gives the results obtained from the tuber progeny of some of the plants from the groups in Table 3. It is apparent that seedlings localizing virus

Y after the two inoculations, were hypersensitive at both growth stages. The results from seedlings which developed systemic necrosis after the second inoculation are interesting, as they show that this reaction is another phase of localization, and that there is a possibility that nutritional factors caused the variation in the hypersensitive response. The fact that 2 tubers from each of 8 seedlings produced healthy plants indicated that the tissues away from the inoculation sites were unfavourable for the extensive development of virus Y. Further, where extensive invasion of tissues including the tubers had occurred, some tubers or "eyes" were free of virus, the rest developing lethal necrosis which appears to be interchangeable with localization. In Table 4, the lethally necrotic plants (see Plate 1, Fig. 1) grew 4 to 6 in. high and died within 21 days of emergence without forming tubers. Those plants shown which were almost lethally necrotic (see Plate 1, Fig. 2) grew 12 in. high and formed tubers before death within 6 weeks of emergence. Plate 1, Figure 2, also illustrates the fact that one tuber from a previously infected plant can produce a healthy plant, while the other produces an almost lethally necrotic one. The reactions in the Katahdin controls (see Plate 1, Fig. 3) were not as severe as in the two former necrotic types. For comparison, Plate 1, Figure 4, shows the tuber progeny from a seedling which was hypersensitive after both inoculations. The tuber progeny of the Factor controls which developed leaf-drop streak after the first inoculation, produced typical secondary symptoms of rugose mosaic.

These results tend to disprove Roland's (1946) hypothesis that the irregular distribution of virus Y in the tuber progeny of infected plants is due to defective diffusion of the virus and not to a reaction of the host tissues. It appears from the results of the Canberra work that if a young vigorously growing plant gives a localized reaction to a relatively heavy dosage of virus Y by hand inoculation, this is a hypersensitive reaction, even if the localization appears to break down at later growth stages, or at periods such as in autumn and winter when the plant is not growing normally.

(a) *The Distribution of Virus Y in Seedlings developing Systemic Necrosis after the Post-flowering Inoculation*

A number of the plants of seedlings shown in Table 3 as giving a localized reaction at pre-flowering and systemic necrosis following inoculation after flowering were kept. At this stage, although tuber formation was advanced, the plants were still making new growth. Samples consisting of two leaflets with, and two leaflets without, necrotic lesions were taken from 22 of the seedlings, and together with leaflets from Factor and Katahdin check plants used to inoculate half leaves of the seedling 11-84. The two leaflets of every sample were each inoculated at random to a half leaf of seedling 11-84. A potato leaf is considered to be divided longitudinally in halves by the petiole and midrib, so that half the terminal leaflet and the next leaflet on the same side constitute a half leaf. In another experiment, 10 half leaves of 11-84 were inoculated from a seedling with a severe Y mottle, the opposite halves being inoculated from another with a severe systemic necrosis. The results of these two experiments are given in Table 5. Seedling 11-84 is a good indicator plant for virus Y, as it is

immune to virus X, and quickly produces small, well-defined, and easily counted localized lesions after inoculation with Y infected material (see Plate 2, Fig. 2).

The results set out in Table 5 are interesting, as they indicate that in a number of the seedlings which developed systemic necrosis after the second inoculation, virus Y was either in very low concentration or absent when both lesion free and necrotic leaflets were considered. Apparently, in these seedlings, the type of systemic necrosis developed was another phase of the localized

TABLE 5

A NUMBER OF POTATO SEEDLINGS, FACTOR AND KATAHDIN, CLASSIFIED ON THE BASIS OF THE REACTIONS OBTAINED AFTER INOCULATION OF THEIR LEAFLETS TO 11-84

Variety	Number of Lesions per Half Leaf on 11-84 after Inoculation from:	
	Leaflets free of Necrosis	Leaflets with Necrosis
Factor	—	15
Katahdin	5	4
15 seedlings	0	0
7 seedlings	2 to 5	0
Mottled seedling	14	—
Necrotic seedling	—	6

reaction. The effect of the necrotic reaction in reducing virus concentration is shown with the 7 seedlings, which had a medium concentration of virus Y in lesion free leaflets and a very low concentration or absence of this virus in necrotic leaflets. This is further borne out by the results with Factor, Katahdin, and the mottled and necrotic seedlings shown in Table 5. In Factor, which develops a strong mottle as well as necrosis, and in the mottled seedling, virus Y concentration appears to be almost double that in the necrotic reacting Katahdin and the necrotic seedling when 11-84 is used as the indicator of concentration. These results are in agreement with those of Bawden and Kassanis (1946) who showed serologically that mottle reacting types had a higher virus concentration than those giving necrotic reactions.

TABLE 6

NUMBERS OF SEEDLINGS WITH SYSTEMIC NECROSIS CLASSIFIED INTO GROUPS ON THE BASIS OF THE RESULTS OBTAINED AFTER REINOCULATION OF TWO OF THEIR LESION FREE LEAFLETS WITH VIRUS Y

	No Reaction on either Leaflet	Reaction on both Leaflets	Reaction on one Leaflet	Total
Seedlings	9	24	5	38
Factor	4	0	0	4
Katahdin	2	0	0	2

Note.—The number of lesions developing on reinoculated leaflets varied from 10 to 20.

In order to investigate these results further, the seedlings which were kept following the development of systemic necrosis after the post-flowering inoculation, were reinoculated with virus Y on selected lesion free leaflets. Two leaflets were inoculated on each seedling, one on a leaf with all leaflets free of systemic necrosis, and one opposite a leaflet with systemic necrosis. Factor and Katahdin checks used in these experiments were inoculated similarly.

The results obtained in this experiment are given in Table 6, and bear out those in Tables 4 and 5, since it is apparent that the tissues of seedlings hypersensitive at pre-flowering, are also unfavourable to the extensive development of virus Y when inoculated after flowering, even though some systemic invasion occurs at this latter stage. The fact that two-thirds of the seedlings shown in Table 6 developed necrotic reactions on lesion free leaflets on reinoculation indicated absence of virus Y in them, and the inference is that where systemic necrosis resulted, the virus had developed and become localized. In Factor and Katahdin virus Y multiplication was more general.

V. INTERACTION BETWEEN STAGE OF GROWTH AND STRAIN OF VIRUS Y IN RELATION TO THE NECROTIC REACTION OF SEEDLINGS

It is apparent from the results presented so far, that localization of virus Y in the potato seedlings which have been developed is not a clear-cut reaction, and although dependent primarily on certain gene frequencies, the expression of this reaction is influenced by plant metabolism at the time of inoculation. In turn, plant metabolism is influenced by environmental factors like photoperiod and temperature which are varying entities in the ordinary greenhouse during the year. Another factor which can influence the localized reaction is the strain of virus Y used. Recently, Bawden and Kassanis (1947) have demonstrated the existence of strains of virus Y differing in virulence.

(a) *The Effect of Strain on the Concentration of Virus Y in Tobacco Plants*

For the experiments concerning the relation between strains of virus Y and the localized reaction of potato seedlings, the following strains of Y were used: Rothamsted (R), Sheffield (S), 18.2, Victorian Y, and virus C. As explained previously, these were transferred each month to a new batch of young tobacco plants. Effects in this host were observed to vary according to the strain, R and S producing relatively severe symptoms, 18.2 and Victorian Y mild symptoms, and virus C causing hardly discernible symptoms. This variation of symptom expression in tobacco due to the strain of virus Y could result from differences in virus concentration. To test this hypothesis, 22 plants of the seedling indicator 11-84 at the flowering stage and as uniform as possible were selected. Three leaves midway on every plant were chosen and 5 of the 6 half leaves each inoculated

TABLE 7

EFFECT OF STRAIN OF Y ON THE VIRUS CONCENTRATION IN TOBACCO PLANTS, SHOWN BY THE NUMBER OF LESIONS ON HALF LEAVES OF THE POTATO SEEDLING INDICATOR 11-84

Strains of Virus Y	R	S	18.2	Vict. Y	C
Mean number lesions/half leaf	36.60	37.41	59.77	101.27	20.50
Mean transforms	5.93	5.99	7.51	9.85	4.35

Minimum difference in mean transforms for significance at 5% = 0.8029.

at random to one of the 5 strains of virus Y. The tobacco plants from which the strains of Y were taken were of similar size, and had all been inoculated at the same time a month previously. A standardized method of applying the inoculum

with the spatula was used throughout, and the inoculum was prepared by grinding 2 g. of infected tobacco leaf in 40 ml. of phosphate buffer at pH 7. The lesions were counted 10 days after inoculation and the results are given in Table 7.

Under the conditions of this experiment, it can be seen from Table 7, that the more severe R and S strains of Y were in significantly less concentration than the relatively mild 18.2 and Victorian Y. Virus C was in significantly less concentration, and Victorian Y in significantly greater concentration, than the other strains. Such differences in concentration could explain in part the relation between virus Y and the hypersensitive reaction of potato seedlings discussed later. It is apparent that severity of symptoms and virus concentration are not necessarily correlated in tobacco, although virus C with its hardly discernible symptoms in this host was in lowest concentration.

(b) Relation between Strain of Virus Y and the Localized Reaction in 50 Selected Seedlings

For this experiment, 50 of the potato seedlings used in the previous experiment were selected. Of these, 24 had localized reactions after pre- and post-flowering inoculations with Victorian Y in the 1946-47 season, and 26 had reacted with systemic necrosis after the pre-flowering inoculation (see Tables 1 and 3). The R, S, 18.2, Victorian Y strains, and a mixture of the first three, were each inoculated separately into different plants of every seedling. The first inoculation was carried out in October 1947 as soon as the first two leaves had expanded sufficiently after emergence. Inoculation at this early growth stage did not decrease the percentage of seedlings with a localized reaction. Those with localized reactions were given a second inoculation at the post-flowering growth stage some 6 to 8 weeks later. The localized reactors from this experiment were harvested and planted again early in March 1948, when they emerged free from virus Y. When 9 to 12 in. high, they were reinoculated with the strain or mixture of strains used on them in the previous October inoculation. A second inoculation was given the plants a month later. It was difficult to separate the different results of the two last inoculations as the plants, although green, grew slowly and tended to mature quickly owing to the shortening photoperiod of autumn, and Y reactions appeared slowly. The results are given in Table 8.

In columns A and B, where one of the plants was late in emerging or weak growing, the result from the more normal duplicate was recorded. It was noticeable that weak plants tended to react with lethal necrosis where the duplicate gave a localized reaction, which is in accord with the previous results. In column C, a seedling was recorded if it had one or more plants which localized virus Y. No seedling at the autumn inoculation gave a localized reaction in all of the 2-5 replicates, showing that the hypersensitive seedlings discussed in this paper will develop a type of systemic necrosis if given the right conditions. The plants which developed systemic necrosis at the autumn inoculation did not have typical symptoms involving the leaves, as the necrosis was confined mainly to the stem.

The results shown in Table 8 do not detract from the general principle of virus Y hypersensitivity propounded in this and previous papers (Hutton and Bald 1945; Hutton 1945, 1946), as seedlings which have reacted hypersensitively at the pre-flowering stage when growing normally in the early summer, will, in the majority of cases give a localized reaction or a light systemic necrosis after flowering. The latter reaction, which can also occur in hypersensitive seedlings inoculated under unfavourable environmental conditions, results, as shown previously, in most of the tuber progeny being healthy or lethally necrotic.

TABLE 8
NUMBER OF SEEDLINGS IN A GROUP OF FIFTY WHICH GAVE LOCALIZED REACTIONS AFTER INOCULATION IN THE SUMMER AND AUTUMN WITH DIFFERENT STRAINS OF VIRUS Y

Strain of Y	Summer 1947-48 Number of Seedlings in Batch of 50 with Localized Reactions			Autumn 1948 Number of Seedlings from B with Localized Reacting Plants
	A	B	Differ- ence	C
	Inoculation at Emergence	Inoculation at Post-flowering		Inoculations March and April 1948
R	36	32	4	13
S	28	18	10	6
18.2	25	18	7	11
Victorian	21	20	1	4
R + S + 18.2	34	28	6	11

Note.—Columns A and B result from inoculation of duplicate plants of each seedling, column C from 2-5 plants of each.

The influence of strain of Y on the numbers of seedlings giving a localized reaction is clearly shown in Table 8. The tendency for R, the most virulent of the strains, to give a localized reaction in the greatest number of seedlings is interesting. This influence is also apparent where the mixture of R, S, and 18.2 was used, the R strain appearing to have eliminated in some way the effects of the S and 18.2 strains, so that the number of localized reactors for the mixture tends to follow the R pattern. The numbers of seedlings localizing the other strains are much the same, although it is of interest that only one seedling developed systemic necrosis at post-flowering with the Victorian strain. This latter fact may be of significance as the 20 seedlings hypersensitive to Victorian Y, after the two summer inoculations, came from the group shown in Table 3 as giving localized reactions in the 1945-46 and 1946-47 seasons after pre- and post-flowering inoculations. It is difficult to correlate the results of Table 8 with those of Table 7. Apparently, other biochemical characteristics, apart from concentration differences, determine the relationship between strain of virus Y and hypersensitivity in potato seedlings.

In order to examine the strain effect more closely, the results from the 20 seedlings which localized Victorian Y in the summer on both occasions, as shown in Table 8, were compared with those from plants of the same seedlings inoculated with the other strains of Y.

It can be seen from Table 9 that the number of seedlings previously found hypersensitive to Victorian Y and localizing the other strains in this experiment varied, the R strain being localized by the greatest number. Almost half the seedlings of the Group in Table 9 gave localized reactions to the other three strains of Y, whereas only one seedling did not have a localized reaction to any of the other three. It is apparent that strain is an important factor determining whether a seedling will be hypersensitive or not, and that a seedling localizing one strain has about a one in two chance of localizing a different strain. The check plants of the varieties Factor and Katahdin used in the experiment of

TABLE 9

NUMBERS OF SEEDLINGS FROM THE GROUP OF TWENTY, WHICH LOCALIZED VICTORIAN Y AT BOTH SUMMER INOCULATIONS, LOCALIZING THE OTHER STRAINS OF VIRUS Y

Localizing R, S, & 18.2		Localizing R		Localizing S		Localizing 18.2		Not Localizing R, S, & 18.2	
1st Inocn.	2nd Inocn.	1st Inocn.	2nd Inocn.	1st Inocn.	2nd Inocn.	1st Inocn.	2nd Inocn.	1st Inocn.	2nd Inocn.
10	6	17	17	12	7	15	11	1	1

Note.—Localizing R, S, and 18.2 in the first and last columns refers to each separately, and not the mixture.

Table 8 gave symptoms depending largely on the strain of Y used. The R and Victorian strains caused the development of leaf-drop streak after the first inoculation in Factor, whereas Katahdin localized these strains. After the second inoculation, Katahdin developed systemic necrosis with the Victorian strain, but localized the R strain. With the S and 18.2 strains, Factor and Katahdin developed after the first inoculation a strong mottle which was more pronounced with the S strain.

(c) *Second Experiment on the Relation between Strain of Virus Y and the Hypersensitivity of Potato Seedlings*

In view of the influence of the strain of virus Y on the localized reaction of seedlings, a second experiment involving fewer seedlings and more treatments was made during the same season as the previous experiment. Thirteen of the seedlings from the 50 used in the previous experiment, and Katahdin, Sequoia, and Factor plants were hand inoculated with the 4 strains of Y which were used separately, and as a mixture. In addition, virus C was used on its own, and side grafting with Epicures containing R and 18.2 Y respectively was done. The mixture of Y strains was obtained by grinding together leaves from different tobacco plants, each carrying one of the strains.

The seedlings selected for this experiment were those which gave a localized reaction the greatest number of times in the previous experiment quoted in Table 8 where each seedling had a chance of giving a localized reaction on 10 occasions, since each seedling was represented by 5 plants which were inoculated twice.

Table 10 gives the numbers of localized reacting seedlings on the different numbers of occasions. The 13 seedlings selected for the second experiment came from the 14 which gave a localized reaction on 8, 9, or 10 occasions in Table 10.

TABLE 10
THE NUMBER OF LOCALIZED REACTING SEEDLINGS ON THE DIFFERENT NUMBERS OF OCCASIONS
IN TABLE 8

Number of Localized Reactions out of 10												Total
No. of seedlings	0	1	2	3	4	5	6	7	8	9	10	
	7	1	3	5	6	3	4	7	5	4	5	50

The results of the experiment are given in Table 11, where the symptoms following the second or post-flowering inoculation and grafting are given, as well as the symptoms in the vegetative progeny of the tubers harvested from the inoculated and grafted plants.

At the first or pre-flowering inoculation, the seedlings Katahdin and Sequoia gave a localized reaction except where lethal necrosis occurred, as indicated in Table 10. Factor gave the usual leaf-drop streak or mottle after the first inoculation with the virus Y strains, and a localized reaction at both inoculations to virus C. After the post-flowering inoculation, Katahdin and Sequoia developed lethal necrosis, systemic necrosis, or leaf-drop streak, depending on the strain of virus Y used.

At the second inoculation, the seedlings with few exceptions gave a localized reaction, lethal necrosis, or a light systemic necrosis, and these reactions resulted in the tuber progeny being healthy or lethally necrotic, showing as before (Table 4) that these reactions were different phases of hypersensitivity. The results in Table 11 from the tuber progeny follow from the planting of 2 tubers from each inoculated plant, and where the results from the two differed it is indicated in the table. In the R, 18.2, Victorian Y, and 4 mixed strain series, 27-41 and 30-39, 27-41, 24-10 and 30-39, and 27-41 and 28-86, respectively, produced healthy plants from the tuber progeny of lethally necrotic plants. Seedling 11-76 and Sequoia are interesting in the R series, as one of the tuber progeny was healthy, and the other lethally necrotic, showing that the apparently localized reaction after the second inoculation allowed the virus to travel down to the tubers. At planting, it was interesting to note that rotting due to necrosis occurred only in tuber progeny of the R series.

When the results of Table 11 were compared with those obtained earlier the same season with the same seedlings, the tendency was for fewer localized reactions at the second inoculation, and more lethal and light systemic necrotic reactions indicating that the level of plant metabolism in the late summer was less conducive to a completely localized reaction. With some seedlings too, like 11-76, 11-84, and 11-158 in the S series, an apparently non-hypersensitive type of reaction was obtained. In most of these instances, the type of leaf-drop streak was more severe than that obtained in Y infected Factor. This has happened before with

TABLE 11
RESULTS FROM THE SECOND OR POST-FLOWERING INOCULATION, AND GRAFTING OF THIRTEEN SELECTED SEEDLINGS AND THREE VARIETIES WITH VARIOUS STRAINS OF VIRUS Y, AND THE SYMPTOMS OBTAINED IN THEIR TUBER PROGENY

Hybrid	R		S		18.2		Vict. Y		4 Mixed		C		R		18.2.	
	2nd Inoc.	Tuber Progeny	2nd Inoc.	Tuber Progeny	2nd Inoc.	Tuber Progeny	2nd Inoc.	Tuber Progeny	2nd Inoc.	Tuber Progeny	2nd Inoc.	Tuber Progeny	Graft	Tuber Progeny	Graft	Tuber Progeny
2-404	L	H	Leth	-	LSN	H	L	H	LSN	H	L	H	Leth	-	Leth	Leth
11-76	L	H, Leth ²	LDS	LDS	LDS	LDS	Leth	Leth ²	LDS	LDS	Leth ¹	Leth ²	Leth	-	Leth	Leth
11-84	L	H	LDS	LDS	Leth ¹	Leth	Leth	Leth ²	Leth	Leth	LSN	H	Leth	H	Leth	Leth ²
11-158	Leth	Leth	LDS	LDS	Leth	Leth	Leth	Leth	Leth	Leth	L	H	Leth	-	Leth	Leth
13-4	LSN	H	L	H	Leth ¹	Leth	Leth ¹	-	L	H	Leth ¹	Leth ²	Leth	-	Leth	-
19-1	LSN	H	LSN	H	LSN	H	LSN	H	LSN	H	LSN	-	Leth	-	LSN	H
23-9	L	H	L	H	L	H	L	H	L	H	L	H	Leth	-	Leth	LDS
24-10	LSN	H	LSN	H	LSN	H	Leth	H	Leth	Leth	Leth	-	Leth	Leth ²	Leth	Leth
27-41	Leth	H	LSN	H	Leth	H	Leth	Leth	Leth ¹	H	Leth	Leth	Leth	-	Leth	Leth
28-86	LSN	H	SN	Mottle	LDS	LDS	LDS	LDS	Leth	H	L	H	Leth	H	LDS	LDS
30-39	Leth	H	SN	-	SN	SN	Leth.	H	Leth	Leth	L	H	-	-	Leth	-
32-164	LSN	H	SN	Mottle	L	H	LSN	Leth ²	L	H	L	H	Leth	H	Leth	-
34-86	Leth	-	Leth ¹	Leth	Leth ¹	Leth	Leth	Leth	Leth ¹	Leth	L	H	Leth	-	Leth	Leth
Katahdin	Leth	Leth	SN	Mottle	SN	Mottle	Leth	H, LDS	Leth ¹	Leth	SN	Rugose	Leth	Leth	Leth	LDS
Sequoia	Leth	H, Leth	SN	LDS	SN	Mottle	LDS	LDS	LDS	LDS	SN	Mottle	LDS	LDS	Leth	-
Factor	LDS	Mottle	Mottle	Mottle	LDS	-	LDS	Mottle	LDS	LDS	L	H	LDS	LDS	LDS	LDS

H = healthy; L = localized necrotic reaction; Leth = lethal necrotic reaction. Seedlings marked Leth¹ reacted with lethal necrosis after the first or pre-flowering inoculation, those marked Leth² did not emerge owing to rotting of the tuber or death of all the "eyes."
SN = systemic necrosis of most of leaflets without leaf drop; LSN = light systemic necrosis; LDS = typical leaf-drop streak.

seedlings which are usually hypersensitive, and the indications are that if hypersensitive seedlings are tested repeatedly at different times and under a wide enough range of conditions, their hypersensitivity occasionally appears to break down. Katahdin frequently reacts with severe leaf-drop streak, being less sensitive than most of the selected seedlings.

The results with virus C in Table 11 paralleled those obtained with the other virus Y strains. When the graft reactions are examined, there is little difference between the results from the R and 18.2 strains, and generally hypersensitivity of seedlings has been expressed by lethal necrosis (see Plate 2, Fig. 1), and where there were tuber progeny, some have emerged healthy. Grafting has not provided more information than hand inoculation, in fact hand inoculation at different growth stages followed by an examination of the tuber progeny will give a more accurate assessment of hypersensitivity to Y than grafting.

As the seedlings shown in Table 11 were specially selected, this experiment shows in a more limited fashion than the earlier, the effect of strain on hypersensitivity. However, it emphasizes how seedlings with hypersensitive reactions to all strains of Y can be developed from basic material like Katahdin.

VI. THE RELATION BETWEEN TYPE OF INOCULATION AND REACTION TO VIRUS Y IN SEEDLINGS

(a) *Hand Inoculation of Leaves compared with Hand Inoculation of Stems*

In the field, aphids, particularly *Macrosiphum gei*, often feed on the young stem tissue at the tips of stems. In view of this, and the fact that comparisons between the Y sensitivity of leaf and stem tissues had not been made, an experiment was done in the 1947-48 season to determine what effect hand inoculation of stems and leaves had on the respective final plant reactions to virus Y.

TABLE 12
COMPARISON BETWEEN THE REACTIONS FOLLOWING HAND INOCULATION OF STEMS AND LEAVES OF POTATO SEEDLINGS WITH VIRUS Y

Reaction of Inoculated Tissue	Total Number of Plants		Final Plant Reaction	Total Number of Plants	
	Stem Inoculation	Leaf Inoculation		Stem Inoculation	Leaf Inoculation
No necrosis	25	0	Free of Y	17	16
Light necrosis	14	8	Systemic* necrosis	27	28
Medium necrosis	3	17			
Heavy necrosis	2	19			

* Plants with systemic necrosis were also showing a mottle in a number of instances.

Duplicate plants of a number of seedlings were inoculated with one of the following: R, S, 18.2 strains of Y, or the three strains mixed. The leaves of one plant were hand inoculated in the usual way, and the stem of the other plant, after dusting with carborundum, was inoculated by rubbing between the thumb and

forefinger dipped in inoculum. The seedlings came from a batch of 20, including both localized and systemic necrotic reactors, the duplicates of which were allocated to the different strain treatments according to the number of plants of each available. The results showed that the strain of Y used had no effect on the comparisons between stem and leaf inoculation, so the results were summated and are given in Table 12.

Table 12 shows that over half the plants did not give a visible necrosis after stem inoculation, whereas all the duplicates reacted necrotically on inoculated leaves. It is doubtful whether this indicates that stem tissue is less sensitive than leaf tissue, as the final plant reactions were much the same irrespective of the method of inoculation.

(b) *Hand Inoculation compared with Aphid Inoculation*

In the 1946-47 season, duplicate plants of 100 seedlings which had been found to include localized, lethal necrotic, systemic necrotic, and mottle reactors to hand inoculation with Victorian Y in the greenhouse, were selected for aphid inoculation. Factor and Katahdin plants were included as checks. The plants were aphid inoculated in the greenhouse when they were at the flowering stage, using *Myzus persicae* under the optimal conditions described by Watson and Roberts (1939). A leaflet on each plant was tagged and inoculated with 4 aphids which had fed for 4 minutes on the underside of a tobacco leaf infected with Victorian Y. Aphid inoculations were carried out in the morning, and the aphids were killed by fumigation in the late afternoon some 5 hours later.

Each week, the tagged leaflets and the plants were examined for virus Y symptoms. After 8 weeks, the 47 plants which exhibited no leaf or plant symptoms were discarded, and the results from those which reacted were analysed and are given in Table 13. Four out of six Factor checks developed leaf-drop streak, and three of the six Katahdin checks reacted, two with a localized reaction, and one with systemic necrosis.

TABLE 13

COMPARISON BETWEEN THE HAND AND APHID INOCULATION RESULTS FROM DUPLICATES OF THE SAME SEEDLINGS

Reaction to Hand Inoculation	Number of Hybrids	Results from Aphid Inoculation			
		Localized	Lethal Necrosis	Systemic Necrosis	Mottle
Localized	19	17	0	2	0
Lethal necrosis	2	1	1	0	0
Systemic necrosis	25	10	0	15	0
Mottle	7	0	0	0	7

Table 13 shows a fair correspondence between hand and aphid inoculation results. That 40 per cent. of the systemic necrotic reactors to hand inoculation gave localized reactions after aphid inoculation suggests that with the lower virus dosages applied in the latter method the virus tends to become inactivated by the necrotic reaction of the inoculated tissues. With the more concentrated virus dosages applied in hand inoculation, only the more complete and rapid localized

reactions are capable of preventing systemic invasion by virus Y. It is possible that the resistance to initial infection of the variety Katahdin to virus Y, as shown by Bawden and Kassanis (1946), is due to a balance between virus dosage and the necrotic reaction of this variety.

The general tendency as shown throughout this study is for the necrotic reaction to inhibit the development of virus Y, and Table 13 indicates the effect that virus dosage has on this relationship. This has already been observed by Schultz, Stevenson, and Akeley (1947) in relation to the field infection of varieties. It is apparent that the level of necrosis required for field resistance will vary according to the density of virus-infected aphids present.

In the experiment of Table 13 it is possible that a number of the 47 seedlings which apparently escaped aphid infection had necrotic reactions which were so minute as to escape detection. Where visible necrotic reactions resulted, these were often no larger than a pin's head and were difficult to find. In view of this, and the possibility of virus dosages influencing the field immunity of a variety or seedling, it appears of greater value to study the development of hypersensitivity in seedling material by means of hand inoculation in the greenhouse. Under these conditions, a high and relatively constant dosage of virus Y can be applied, so that a more accurate assessment of the true hypersensitivity of any particular seedling can be made.

VII. HISTOLOGICAL CHANGES IN THE STEMS OF VIRUS Y INFECTED PLANTS

Quanjer (1931) and Bawden (1932) examined microscopically the stems of potato plants affected with acropetal necrosis or leaf-drop streak caused by virus Y, and found in the collenchyma necrotic areas which sometimes extended into the rest of the cortex but not to the vascular bundles. Bawden (1932) found that the epidermis and periderm occasionally showed necrotic areas, and that the internal necrosis characteristic of virus Y was usually present immediately below nodes from which leaves had fallen. He found that acropetal necrosis spread perpendicularly in the stem and produced dark streaks visible below the epidermis. Bawden also found a correlation between external and internal symptoms, varieties like Up to Date (Factor) reacting with leaf-drop streak having internal necrosis, whereas Epicure and varieties reacting with a mottle having no internal necrosis, thus showing the necrotic reaction to be dependent on variety.

The work described in this paper confirms Bawden's (1932) findings and presents further results on the distribution of internal necrotic areas, particularly in hypersensitive reactors to virus Y. It was made possible by using a modification of the method used by Sheffield (1943) in the diagnosis of potato leaf roll. Wilson (1948) has pointed out that phloem necrosis in leaf-roll affected potato plants is more easily distinguished if concentrated hydrochloric acid is used in Sheffield's method. Work at Canberra has shown that 40 per cent. sulphuric acid (v/v) used in conjunction with 2 per cent. phloroglucinol (w/v) in 50 per cent. alcohol (v/v) is a reliable combination for distinguishing necrotic areas in hand cut transverse stem sections, whether they be due to virus Y or leaf roll. With this

modification, necrotic areas due to virus Y stain a bright red colour, and those due to leaf roll a pink colour.

When the stem sections of plants which have reacted necrotically to virus Y are treated as described and examined microscopically, a bright red staining of cell walls in the necrotic region is visible. Usually the collenchyma with the thickening at cell corners is characteristically outlined and often the staining of cell walls continues into the rest of the cortex (see Plate 2, Fig. 3), and in severely affected plants phloem tissue is involved as well. Cell distortion often occurs when the thin walled cortical cells and the phloem are affected. In lethal necrotic types, cell walls often in large areas of the stem are stained from the epidermis to the pith, involving secondary phloem as well as the internal and external primary phloem. With such plants, there is little likelihood of confusing the reaction with leaf roll, as the phloem is only involved in Y necrosis when other tissues are heavily infected. It was not possible to determine whether the xylem was affected by virus Y as it showed no distortion and stained red in healthy plants.

Most of the observations on plants reacting necrotically to virus Y were made on material grown in the greenhouse during 1948. One group of plants examined was grown from tubers of seedlings and the varieties Factor, Katahdin, and Sequoia infected the year previously with the various strains of virus Y. On emergence, the plants of this group were either healthy or had mottle, leaf-drop streak, or lethal necrotic symptoms. Those free of virus Y symptoms had no internal necrotic areas in transverse sections hand cut progressively from the base to the tip. Thirty-three plants of the group had necrotic areas in various portions of the stem, the distribution and extent of necrosis being dependent on the severity of the symptoms. Table 14 gives the different distributions of the plants within the mottle, leaf-drop streak, and lethal necrotic groups respectively, according to the stain reactions found in the base, mid, and tip stem sections. Factor plants occurred in the mottle group, as the secondary Y symptoms in this variety were a rugose mottle which was also the secondary symptom in the other varieties with some of the virus Y strains used.

In Table 14, when basal stem sections are considered, the majority of the plants in the mottle and leaf-drop streak groups had a light staining in the collenchyma and the rest of the cortex, whereas in the lethal necrotic group most of the plants were heavily stained in this region, and in over one-third of them staining extended into the pith. Where staining was intense, phloem tissue was involved in the stem sector affected. The results for the mid-stem sections show a similar gradation, half of the plants of the mottle group being unstained and the rest lightly stained, whereas in the leaf-drop streak group over half were heavily stained, and in the lethal necrotic plants the majority were stained extensively from the epidermis to the pith. In the stem tip sections staining was absent in the mottle group, and absent or light in the leaf-drop streak plants, whereas the majority were stained in the lethal necrotic group, half being intensively stained.

These results are an index of the varying sensitivity to virus Y found among varieties and seedlings. There appears to be a clear distinction between the mottle group on the one hand and the lethal necrotic group on the other, the leaf-drop streak plants having internal necrotic symptoms intermediate between these two. Table 14 also indicates that in the lethally necrotic plants the tissue is so sensitive that a systemic invasion of the virus results in necrosis and death wherever the virus develops, whereas in the tolerant mottle types virus development can occur in various sites without observable necrosis of the tissue.

TABLE 14

NUMBERS OF PLANTS WITH MOTTLE, LEAF-DROP STREAK, AND LETHAL NECROSIS CLASSIFIED ON THE BASIS OF THE INTERNAL STAINING REACTION OF THEIR STEMS TO VIRUS Y

Type and Extent of Staining* observed in Stem Sections	Mottle Sections at:			Leaf-drop Streak Sections at:			Lethal Necrosis Sections at:		
	Base	Mid	Tip	Base	Mid	Tip	Base	Mid	Tip
No staining	3	5	10	0	0	5	0	0	2
Light staining collenchyma and cortex	6	4	0	7	4	5	3	1	4
Heavy staining collenchyma and cortex	1	0	0	3	3	0	6	3	1
Heavy staining from epidermis to pith	0	1	0	0	3	0	4	9	6
Total plants	10			10			13		

* The observations on staining refer to tissues other than the xylem, which is always stained with the method used. "No staining" refers to absence of stained areas in tissues other than the xylem; "light staining" means relatively small lightly stained areas; and "heavy staining" refers to extensive deeply stained areas.

A further set of observations was made on a group of seedlings, the plants of which had given a localized reaction after hand inoculation of leaves in some, and stems in others with the various strains of virus Y. The numbers of localized reacting plants examined varied owing to the effect of strain, and also owing to the number of duplicates of the seedlings available initially for inoculation. Stem sections were taken from just above to just below the point of attachment of the inoculated leaf, and through the inoculation site where the stem was inoculated. The results are summarized in Table 15.

All the plants of the seedlings shown in Table 15 were subsequently proved free of virus Y when the tubers harvested from them were grown on in the greenhouse. The fact that a staining reaction occurred in stem sections of a varying proportion of seedlings which gave an apparently localized reaction to virus Y in inoculated leaves is interesting. The necrotic areas in the stem were stained

relatively lightly, but were clearly defined, and in approximately half the plants, occurred in the collenchyma and rest of the cortex, and in the others extended from the collenchyma to the pith. These results indicate that the localizing reaction is not always completed in the inoculated leaf, but in stem tissue. The extent to which this occurs is probably dependent on the nutritional status of the plant at the time of inoculation. That staining, and hence necrotic tissue, can occur away from the inoculation site in localized reactors further substantiates the findings of Tables 4 and 11, in which it was shown that a light systemic necrosis after the post-flowering inoculation, in localized reacting seedlings at pre-flowering, was a variation of the localized reaction.

TABLE 15

NUMBERS OF LOCALIZED REACTING SEEDLINGS CLASSIFIED ACCORDING TO INTERNAL STAINING IN THE REGION OF INOCULATION AND IN RELATION TO THE Y STRAINS USED

Strain of Virus Y Used	Leaf Inoculation Number of Seedlings with:		Stem Inoculation Number of Seedlings with:	
	No Staining in the Region of Inoculation	Staining of Collenchyma and Cortex or Collenchyma to the Pith	No Staining in the Region of Inoculation	Extensive Staining from the Epidermis to the Pith in the Region of Stem Necrosis
R	15	9	5	5
S	8	8	3	4
18.2	19	2	5	1
R + S + 18.2	9	7	6	5

Although not highly significant, it is interesting that the mildest Y strain, 18.2, gave the highest proportion of the seedlings free of stained necrotic areas away from the inoculated leaves. In the stem inoculation results, those seedlings which had no internal staining were free of stem necroses, so it is probable that the localization sites were not easily discernible in the sections examined microscopically. Where stem necroses were visible externally, internal staining was intense and extended from the epidermis to the pith and involved the phloem tissue as well. It is apparent that stem tissue as well as leaf tissue can react in a hypersensitive fashion. In the instances where internal necrotic areas followed stem inoculation, staining was restricted to the inoculation sites and the virus must have been localized in these necrotic areas as the tuber progeny of the plants were healthy.

The staining results give a clearer understanding of the type of localized reaction which occurs in many of the seedlings developed. Since stem tissue, as well as leaf tissue, can react hypersensitively it is apparent that if the virus travels from the inoculated leaf it can still be localized in the stem before it reaches the tuber. In lethal necrotic types, which form tubers from which healthy plants are at times produced, it is probable that the virus does not diffuse to all the "eyes." When the virus reaches the "eyes," they are either killed or produce lethally necrotic plants. In view of the staining results, it is suggested that the use of the staining technique in conjunction with hand inoculation methods would be

useful in the selection of seedlings with promising reactions to virus Y from hybrid progenies.

VIII. DISCUSSION

This study has demonstrated how hypersensitivity to virus Y and its strains can be developed from common varieties with a severe necrotic reaction to this virus. It has been made possible by the heterozygous nature of the potato, which has allowed the accumulation by the selection methods used, of genes conditioning necrosis of seedlings to virus Y. This has resulted in seedlings with gene frequencies high enough to produce hypersensitivity to a range of virus Y strains. Even though new strains of virus Y may arise to which such seedlings are no longer hypersensitive, this work has indicated how such a problem could be overcome.

In view of these results, hypersensitivity to virus Y can now be added to the characteristics of new potato varieties. That this attribute can be combined with other desirable features has been demonstrated, as 1.2 per cent. of the seedlings from the progenies of carefully planned potato crosses had high yield, desirable tuber characters, immunity to virus X, and hypersensitivity to viruses A and Y. Since relatively low and fluctuating aphid populations have occurred in all the field trials made, it has not been possible accurately to assess the value of the new seedlings in comparison with varieties like Katahdin and Snowflake. Virus dosage is a varying entity under these conditions, so comparisons have been made in the greenhouse where a greater degree of control can be exercised over this and other factors.

It is apparent from this study that the type of sensitive reaction to virus Y present in the seedlings is not similar to the hypersensitivity of Epicure and other varieties to virus X, as this latter reaction is not recorded as being influenced by nutritional or other conditions. Further, it has been shown (Hutton 1945) that virus Y sensitivity in the seedlings is inherited as a recessive character. Cockerham and M'Ghee (1947) have shown that the hypersensitivity to virus Y discovered in *Solanum simplicifolium* is due to a unit dominant gene, so this reaction is apparently governed by different factors from those reported in this study.

In tolerant types reacting with a mottle to virus Y, this virus can move freely and build up a high concentration, whereas if the tissues of a variety react necrotically, the virus, if it gains entrance, is restricted in its movement and development. With tolerant types, infection and kind of reaction do not appear to be greatly influenced by environmental and other factors. On the other hand, in hypersensitive seedlings, the expression of the localized reaction is influenced by growth conditions. The localized reaction is rapid and confined to the inoculation site when plants are young and actively growing. Conditions which lower the metabolic activity of such plants can allow the ingress of the virus into tissues distant from the inoculation site, but since stem as well as leaf tissue is sensitive, the virus is usually localized before it reaches the tubers. Reactions like these give some understanding of the physiological basis for intolerance to viruses. Intolerance to viruses offers most promise for the solution of current virus problems in the potato crop.

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X. REFERENCES

- BALD, J. G., and NORRIS, D. O. (1945).—Virus C from an old Australian variety of potato. *Phytopathology* **35**: 591-7.
- BALD, J. G., and PUGSLEY, A. T. (1941).—The main virus diseases of the potato in Victoria. Coun. Sci. Industr. Res. Aust., Pamph. No. 110, p. 17.
- BAWDEN, F. C. (1932).—A study on the histological changes resulting from certain virus infections of the potato. *Proc. Roy. Soc. B* **111**: 74-85.
- BAWDEN, F. C., and KASSANIS, B. (1946).—Varietal differences in susceptibility to potato virus Y. *Ann. Appl. Biol.* **33**: 46-50.
- BAWDEN, F. C., and KASSANIS, B. (1947).—The behaviour of some naturally occurring strains of potato virus Y. *Ibid.* **34**: 503-16.
- BAWDEN, F. C., and SHEFFIELD, F. M. L. (1944).—The relationships of some viruses causing necrotic diseases of the potato. *Ibid.* **31**: 33-40.
- CADMAN, C. H. (1942).—Autotetraploid inheritance in the potato: some new evidence. *J. Genet.* **44**: 33-52.
- COCKERHAM, G. (1943).—The reactions of potato varieties to viruses X, A, B, and C. *Ann. Appl. Biol.* **30**: 338-44.
- COCKERHAM, G. (1945).—Some genetical aspects of resistance to potato viruses. *Ibid.* **32**: 280.
- COCKERHAM, G., and M'GHEE, T. M. R. (1947).—Rep. Scottish Soc. Res. Plant Breed., 24th July 1947.—Virus diseases, pp. 17-8.
- HUTTON, E. M. (1945).—The relationship between necrosis and resistance to virus Y in the potato. II. Some genetical aspects. *J. Coun. Sci. Industr. Res. Aust.* **18**: 219-24.
- HUTTON, E. M. (1946).—Id. III. Interrelation with virus C. *Ibid.* **19**: 273-82.
- HUTTON, E. M., and BALD, J. G. (1945).—Id. I. Greenhouse results. *Ibid.* **18**: 48-52.
- JONES, L. K., and VINCENT, C. L. (1937).—The susceptibility of potatoes to the vein-banding virus. *J. Agric. Res.* **55**: 69-79.
- JONES, L. K., VINCENT, C. L., and BURK, E. F. (1940).—The resistance of progeny of Katahdin potatoes to viroses. *Ibid.* **60**: 631-44.
- QUANJER, H. M. (1931).—The methods of classification of plant viruses, and an attempt to classify and name potato viroses. *Phytopathology* **21**: 577-613.
- ROLAND, G. (1946).—Sur la résistance de défense des variétés de pomme de terre à l'égard du virus Y (*Solanum virus* 2. Orton). *Parasitica* **2**: 89-92.
- SCHULTZ, E. S., CLARK, C. F., BONDE, R., RALEIGH, W. P., and STEVENSON, F. J. (1934).—Resistance of potato to mosaic and other virus diseases. *Phytopathology* **24**: 116-32.
- SCHULTZ, E. S., STEVENSON, F. J., and AKELEY, R. V. (1947).—Resistance of potato to virus Y, the cause of vein-banding mosaic. *Amer. Potato J.* **24**: 413-19.
- SHEFFIELD, F. M. L. (1943).—Value of phloem necrosis in the diagnosis of potato leaf-roll. *Ann. Appl. Biol.* **30**: 131-6.
- STEVENSON, F. J., SCHULTZ, E. S., and CLARK, C. F. (1939).—Inheritance of immunity from virus X (latent mosaic) in the potato. *Phytopathology* **29**: 362-5.
- WATSON, M. A., and ROBERTS, F. M. (1939).—A comparative study of the transmission of *Hyoscyamus virus* 3, potato virus Y, and cucumber virus 1 by the vectors *Myzus persicae*, *M. circumflexus*, and *Macrosiphum gei*. *Proc. Roy. Soc. B* **127**: 543-76.

- WERNER, H. O. (1934).—The effect of a controlled nitrogen supply with different temperatures and photoperiods upon the development of the potato plant. *Coll. Agric., Univ. Neb. Agric. Exp. Sta., Res. Bull. No. 75.*
- WILSON, J. H. (1948).—The use of the phloroglucinol test for diagnosis of leaf-roll in potatoes. *J. Aust. Inst. Agric. Sci.* 14: 76-8.

EXPLANATION OF PLATES 1-2

PLATE 1

- Fig. 1.—Lethally necrotic plants raised from two tubers of a seedling which previously gave a localized reaction to Victorian Y after the pre-flowering inoculation, but systemic necrosis following the post-flowering inoculation.
- Fig. 2.—A plant with almost lethal necrosis and a healthy plant raised from tubers of a seedling which previously gave a localized reaction to Victorian Y after the pre-flowering inoculation, but systemic necrosis following the post-flowering inoculation.
- Fig. 3.—Plants with a severe leaf-drop streak raised from two tubers of a Katahdin plant which gave a localized reaction to Victorian Y after the pre-flowering inoculation, but systemic necrosis following the post-flowering inoculation.
- Fig. 4.—Healthy plants from the two tubers of a seedling which gave a localized reaction after both pre- and post-flowering inoculations with Victorian Y.

PLATE 2

- Fig. 1.—The lethally necrotic reaction in a hypersensitive potato seedling, following a side graft with a Y infected Epicure scion.
- Fig. 2.—Type of localized necrotic reaction in a leaf of the potato seedling indicator plant 11-84 a week after inoculation with virus Y.
- Fig. 3.—Photomicrograph (x 100) of hand cut transverse stem section of a potato plant reacting with systemic necrosis to virus Y, showing the staining reaction in the collenchyma (A) and the rest of the cortex (B) following treatment with 40 per cent. sulphuric acid and 2 per cent. phloroglucinol solution.



Fig. 1



Fig. 2



Fig. 3



Fig. 4



Fig. 1



Fig. 2

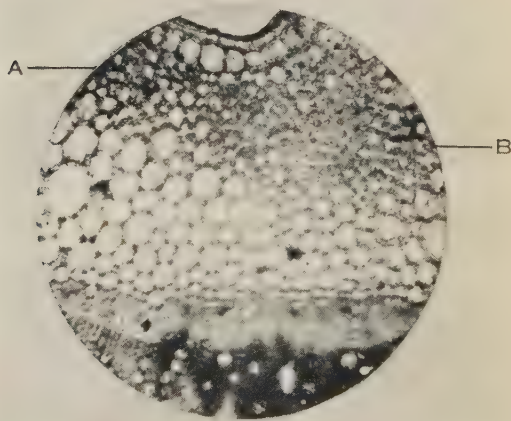


Fig. 3

THE SEPARATION OF STRAINS FROM A VIRUS X COMPLEX BY PASSAGE THROUGH POTATO SEEDLINGS

By E. M. HUTTON*

(Plates 1-4)

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Summary

A high proportion of the potato seedlings from the progenies of crosses between X tolerant parents, Katahdin being common to each cross, gave light to medium necrotic reactions on leaves inoculated with a virus X complex. Localization of virus X was incomplete, and a small percentage of the necrotic reactors acted as strain separators so that masked, mild, severe, and necrotic strains of X were obtained. Strain separation by potato seedlings was influenced by environmental conditions. A small-scale experiment showed the potato seedling technique to be a practicable one for the separation of strains of virus X from a complex. The results provide an explanation for the appearance of new and virulent strains of X in new potato varieties.

Two types of masked X were distinguished, one which maintained protection against a severe strain, and a second which diminished in concentration in an aging host plant so exposing loci for the multiplication of severe strains. Serological tests indicate that the differences between masked and severe strains of X are due to differences in concentration.

I. INTRODUCTION

This investigation was planned initially to test the hypothesis that seedlings hypersensitive to virus X could be obtained from the progeny of crosses between parents tolerant to this virus. It was hoped to demonstrate the origin of the field immune reaction to virus X of varieties like Arran Crest, Epicure, and King Edward (Cockerham 1943). During the summer of 1945 hybrids from various progenies were selected for their necrotic reaction and apparent resistance to virus X. Further work with the selected hybrids showed that they were susceptible to this virus and that infection had caused the original virus X complex to be sorted into strains of different severity. The data presented in this paper deal with this process of separation and the reactions of the strains in different hosts.

Strains of virus X have been dealt with by a number of workers, notably Salaman (1938) who developed a technique of strain separation involving the removal, with a fine punch, of selected tissue from infected tobacco plants. Bald and White (1942), and Bald (1943) have dealt with the effect of mixtures of X strains on potato and *Datura stramonium*. Most strains of virus X noted in the literature have arisen naturally in field-grown potatoes (Clinch 1944; Larson 1943, 1947).

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II. MATERIALS AND METHODS

The potato seedlings used in this study were taken at random from the progeny of a number of crosses involving only virus X tolerant parents. They were grown in the greenhouse, and when about 12 inches high and growing vigorously, two leaves of each seedling were tagged and dusted lightly with an "extra fine" grade of carborundum and then inoculated with virus X using a ground glass spatula. The inoculum was prepared by grinding Brownell potato leaves at the constant proportion of 1:20 by weight with water. The line of Brownell potato used throughout the experiments was tested previously and found to be free from all viruses except X. Tests for the presence of virus B in the line were made with Epicure and virus-free President (Bawden and Sheffield 1944) and gave a negative result. Bald and White (1942) have shown that the Brownell potato carries a mixture of virus X strains differing in severity, the severe strains being in high proportion.

For most of the work, the main indicator plant used was *Datura stramonium*. The presence of masked X in the *Daturas* was indicated by the extent of protection afforded after reinoculation with a severe strain (Salaman 1933). Other indicator plants used at various times in the course of the experiments were the Epicure potato, tomato (*Lycopersicon esculentum*, variety Bonny Best), tobacco (*Nicotiana tabacum* var. *atropurpureum*), *N. glutinosa*, *N. campanulata*, and *Datura metel*.

Serological methods were used in the later stages to supplement the results obtained with the indicator plants. Antiserum was prepared by injecting the clarified sap from frozen young tobacco leaves infected with Brownell X into the marginal ear vein of six-month-old rabbits. A course of nine injections involving a total of 16 ml. of clarified sap was given each rabbit. The precipitin reaction was tested at 37°C. using 0.5 ml. of a 1:5 dilution of the antiserum, and 0.5 ml. of clarified sap from frozen leaves of the plants under test. Under the conditions of the experiments, the 1:5 antiserum dilution gave an immediate precipitin reaction with the clarified sap of Brownell plants, and no result with controls consisting of clarified sap from virus-free Katahdin, tobacco, and *N. campanulata* plants. This dilution proved to be the most satisfactory for distinguishing concentration differences in plants due to strain.

III. PRELIMINARY RESULTS OBTAINED AFTER INOCULATION OF POTATO SEEDLINGS WITH THE BROWNELL VIRUS X COMPLEX

During the summer of the 1945-46 season, 1,200 seedlings were taken at random from the progeny of 13 crosses having only virus X tolerant parents. These were inoculated with Brownell X and the symptoms on the inoculated leaves and the rest of the plant were observed at weekly intervals. The seedlings gave either no reaction on the inoculated leaves or definite necrotic lesions which usually appeared within 14 days. The necrotic reactions of the latter group of seedlings were graded arbitrarily on the number of lesions into five classes, Class 1 averaging a few lesions per leaflet and Class 5 about 100 lesions per

leaflet. Table 1 summarizes these results and shows that the majority of necrotic reacting seedlings gave a light to medium reaction on the inoculated leaves.

TABLE 1

PERCENTAGES OF NECROTIC REACTING SEEDLINGS IN THE VARIOUS CLASSES ACCORDING TO THE NUMBER OF LESIONS ON THE INOCULATED LEAVES

Necrotic Reaction Class	1	2	3	4	5	Total Seedlings
Percentage	25.3	37.2	24.4	11.4	1.7	536

Plate 1, Figures 1 and 2, shows the necrotic reactions on the inoculated leaves of two seedlings from the cross N.Z. Pink Eye x (Snowflake x Katahdin).

Seedlings which initially gave a necrotic reaction could on later examination be placed in one of three classes, those remaining free of systemic symptoms, those developing a definite mottle, or those developing a mottle and systemic necrosis. The results obtained are given in Table 2 where it can be seen that all crosses contained a high percentage of seedlings with a necrotic reaction to virus X. The high percentages of necrotic reactors in the various progenies may have been due to the fact that Katahdin was a parent common to all crosses. Numbers of seedlings with an apparently localized reaction to virus X in the different crosses varied from 5.8 to 39.0 per cent. None of the parents used in the different crosses gave definite necrotic reactions on inoculated leaves, and only a poorly defined systemic mottle after inoculation with Brownell X. The results with the progenies of these parents indicate that factors governing a necrotic reaction and perhaps resistance to virus X are present in tolerant varieties. These factors are probably different from those described by Cadman (1942) as occurring in hypersensitive varieties like Epicure.

The 245 seedlings shown in Table 2 as giving a necrotic reaction in the summer without the subsequent development of a systemic mottle were harvested. Early in June 1946 (mid-winter) all the harvested seedlings were planted in duplicate and observed for mottle symptoms after emergence. Of these, 10 per cent. were rejected because of the development of a definite mottle. A plant of each of the remaining seedlings was inoculated with Brownell X and 14 per cent. of them gave no reaction, whereas the rest gave a definite necrotic reaction on the inoculated leaves. From this it was assumed that those not giving a reaction were carrying mild or masked strains which were protective, and those which gave a definite necrotic reaction were free from X. Accordingly all the X-inoculated potato seedlings were back inoculated to plants of *Datura stramonium* when it was found that all the necrotic reactors now had virus X systemically and the non-reactors mild or masked X. The types of virus X shown by the *Daturas* from the necrotic reactors did not appear to differ markedly from the symptoms of Brownell X on *Datura*. These results showed that in the summer series of inoculations a proportion of the seedlings in some way caused a sorting out of mild or attenuated strains from the original Brownell complex.

In view of the results obtained with the seedlings it was decided to see whether the work could be repeated. Accordingly, all the uninoculated and non-infected duplicate material from the seedlings was planted and inoculated

TABLE 2
PERCENTAGES OF SEEDLINGS FROM TOLERANT PARENTS IN THE DIFFERENT REACTION CLASSES TO VIRUS X

Cross	Parentage	Per Cent. Plants with no Necrotic Reaction on Inoculated Leaves	Per Cent. Plants with Necrotic Reactions on Inoculated Leaves			Total Number
			Systemic Mottle	Systemic Mottle and Necrosis	Without Systemic Mottle	
12, 23	New Zealand Pink Eye					
	x (Snowflake x Katahdin) ¹	34.6	19.4	21.2	24.8	165
21	Delaware					
	x (Snowflake x Katahdin) ²	62.8	5.3	14.9	17.0	94
25	Early Carman					
	x (Snowflake x Katahdin) ²	41.0	1.9	18.1	39.0	105
26	Mohawk					
	x (Snowflake x Katahdin) ²	62.2	1.5	15.2	21.1	66
27	(Brown's River x Katahdin)					
	x (Snowflake x Katahdin) ²	71.0	5.5	16.4	7.1	55
28	Brownell x Katahdin	50.0	9.7	12.9	27.4	62
29	Factor					
	x (Snowflake x Katahdin) ²	73.5	6.0	6.0	14.5	83
30	Brownell					
	x (Snowflake x Katahdin) ¹	59.5	1.3	20.2	19.0	79
31	Factor					
	x (Brown's River x Katahdin)	55.5	3.0	28.3	13.2	99
32	Snowflake					
	x (Brown's River x Katahdin)	61.6	1.9	17.6	18.9	159
33	Carman					
	x (Brown's River x Katahdin)	46.0	6.4	14.3	33.3	63
34	(Snowflake x Katahdin) ¹					
	x (Snowflake x Katahdin) ²	65.0	0.0	15.7	19.3	83
35	Bismark					
	x (Brown's River x Katahdin)	56.3	3.4	34.5	5.8	87

Note.—Two different Snowflake x Katahdin seedlings were used as male parents. The same Brown's River x Katahdin seedling parent was used in all cases.

with Brownell X during the autumn of 1947. The seedlings gave necrotic reactions of varying degrees of intensity and were back inoculated to *Datura* plants a month later. Twenty of the *Daturas* showed no symptoms, and reinoculation with Brownell X produced no further symptoms, proving the presence in these of masked or very mild X strains. Most of the other *Daturas* showed symptoms similar to that given by Brownell X, but 12 were selected as

having more severe symptoms. One of the *Daturas* with the more severe symptoms was inoculated from a seedling giving a severe systemic necrosis.

These results showed that the previous attenuation process was not fortuitous and could be repeated, and that none of the necrotic reacting seedlings were true hypersensitives to virus X, as in this series none of them was free from X following inoculation. It also seemed probable that the necrotic reaction was necessary to the attenuation process described. The results obtained previously suggested that under certain conditions, as in midsummer, the necrotic reactors could give a fully localized reaction to virus X. The effect of environmental conditions on the susceptibility of plants to virus has already been noted by Bawden and Roberts (1947). In this connection it appeared from the results that seedlings giving attenuated strains under one set of conditions did not necessarily do so under others, as only six of the seedlings which apparently contained masked X previously were included in the masked X group when the experiment was repeated.

IV. THE EFFECTS ON DIFFERENT HOSTS OF THE X STRAINS OBTAINED FROM NECROTIC REACTING SEEDLING MATERIAL

The 20 *Daturas* with very mild or masked X symptoms and the 12 with severe symptoms resulting from the second or repeat inoculation of the seedlings with Brownell X were inoculated to further *Daturas* and the results observed in comparison with *Daturas* inoculated from Brownell. Of those with severe symptoms, six were discarded as giving similar results to Brownell. Five of the *Daturas* with mild symptoms were also discarded as they developed symptoms similar to that of Brownell X, showing that they still contained in low concentration severe strains which built up after passage through the *Daturas*. The other 15 *Daturas* with masked or mild symptoms when reinoculated with Brownell X showed no further development of symptoms, so it was assumed that the strains contained in them were relatively pure.

The remaining 21 *Daturas* containing masked and severe strains of X respectively, in comparison with the standard Brownell X, were each inoculated to two plants of tomato, *Nicotiana glutinosa*, tobacco, and *Datura stramonium*. The results in the four different hosts were in good agreement, *N. glutinosa* tending to distinguish differences more quickly and definitely than the other indicators under the hot summer conditions at the time of this experiment. Plate 2, Figures 1 and 3, shows one of the masked strains in *Datura* and *N. glutinosa* respectively, in comparison with Brownell X in *N. glutinosa* in Plate 2, Figure 2. Plate 3, Figures 1, 2, and 3, shows a severe strain in *Datura*, *N. glutinosa*, and tobacco respectively.

All the strains were then passed through *Daturas* a further six times to see whether there was any change in symptoms. During these passages all the severe strains gave more severe symptoms than the Brownell controls, and all the mild strains retained their mildness, although they could be separated into nine which did not give symptoms and six which gave mild mottles.

The six severe, six of the masked strains, and Brownell X were each inoculated from their respective *Daturas* to two virus-free plants of the following potato varieties — Katahdin, Sebago, Factor, President, U.S.D.A. Seedling 41956, and Epicure. None of the strains caused any reaction in S41956. The masked strains produced very little reaction in any of the other potato varieties, although two of them caused a few lesions on the inoculated leaves of Epicure. In Katahdin, Sebago, Factor, and President the masked strains provided protection from one of the severe strains. All the severe strains produced definite localized lesions in Epicure, and two of them caused severe systemic necrosis and mottle in Katahdin and Sebago, necrosis and strong mottle in President, and a strong mottle only in Factor. Plate 4, Figures 1 and 2, shows the severe systemic necrosis in Sebago and Katahdin respectively as a result of inoculation with one of the two severest strains mentioned. The other four severe strains produced only mottles with occasional necrosis in Katahdin, Sebago, President, and Factor, the mottles, however, being more defined than those resulting from Brownell X in these varieties.

The two severest strains described are similar to the X^N strain of Salaman (1938) whereas the other four severe strains are closest his X^S strain. Of the 15 mild and masked strains, the six which gave mild mottles are close to Salaman's X^G strain, and the nine masked strains are similar to his X^H strain. No attempt was made to classify the strains obtained in this study definitely into Salaman's categories as the main purpose of the experiments was to show how seedling material could be used to separate strains of X from a complex. In addition, the behaviour of the masked strains isolated were of particular interest as some of them behaved differently from the standard X^H strain of Salaman.

One of the necrotic strains, a masked strain, and Brownell X were recovered from Katahdin by inoculation to *Datura* and were then transferred from the *Daturas* to tobacco plants. Six weeks after inoculation, the tobacco plants were inoculated to a series of Katahdin plants when, as before, the necrotic strain produced severe necrosis and mottle, Brownell X a fleeting mottle, and the masked X no observable symptoms. The tobacco plants carrying the necrotic strain developed brown necrotic patches, particularly on the lower leaves, which resulted in the gradual death of some of these leaves. Tubers which were harvested from Katahdin, Sebago, President, and Factor plants inoculated previously with the six severe, six masked, and Brownell X developed plants with substantially the same symptoms as those from which they were originally derived. Plants from the tubers carrying the necrotic strains of X were noticeably weak and spindly in growth and gradually died with a spreading systemic necrosis which developed in large patches.

From these results, it was apparent that some stabilized masked and severe strains of X had been separated from the original Brownell complex by its passage through potato seedlings of varying genetic constitution. The heterozygous nature of the potato ensures that the progeny of any particular cross is almost as variable genetically as seedlings taken from different crosses

at random. It can be postulated that the varying genetic constitution of potato seedlings means a differing physiological response to the strains present in a virus X complex.

V. TECHNIQUE FOR THE SEPARATION OF VIRUS X STRAINS BY THE USE OF POTATO SEEDLINGS

Since the results so far obtained were due to experiments which originally involved 1,200 potato seedlings, it seemed desirable to see whether the same separation of strains could be effected using much smaller numbers. Accordingly, 50 seedlings were taken at random from a cross between two X tolerant seedlings, one from the cross N.Z. Pink Eye x (Snowflake x Katahdin) and the other from the cross (Snowflake x Katahdin). All were inoculated with Brownell X from *Datura* in November 1947 (summer) and 12 gave localized lesions on the inoculated leaves. The seedlings were all inoculated to *Datura* plants, when it was found that the seedlings which gave no necrotic reaction on the inoculated leaves, and six of the necrotic reactors produced similar symptoms to Brownell X in the *Daturas*. Five of the *Daturas* from the necrotic reactors were proved to have mild or masked strains, and one appeared to contain a severe strain. The seedlings with no necrotic reaction were discarded, and uninfected duplicate material of the 12 necrotic reactors was inoculated a month later in December 1947. The results obtained are compared with the November experiment in Table 3. It is apparent from these results that it is not possible to predict how a seedling will react with respect to an X complex under different sets of conditions. Table 3 shows that, with the exception of 50-12, 50-48, and 50-58 which

TABLE 3
RESULTS OBTAINED FROM INOCULATING DUPLICATE POTATO SEEDLING MATERIAL
AT TWO DIFFERENT TIMES

Seedling No.	Inoculation with Brownell X		Second Passage Through <i>Daturas</i> from Infected Seedlings	
	Nov. Series	Dec. Series	Nov. Series	Dec. Series
50-2	Systemic necrosis	Localized	+	+
50-4	Severe systemic necrosis	Localized	+	+
50-6	Localized	Localized	+	+
50-10	Localized	Localized	0	+
50-12	Localized	No reaction	+	+
50-14	Severe systemic necrosis	Localized	+	+
50-27	Systemic necrosis	Localized	+	+
50-48	Localized	No reaction	+	+
50-56	Localized	Localized	0	+
50-57	Systemic necrosis	Localized	0	+
50-58	Localized	No reaction	0	0
50-59	Systemic necrosis	Localized	0	0
Brownell	—	—	+	+

Note.—0 = no symptoms on *Datura* after reinoculation with severe X; + + = moderately severe mottle symptoms with some necrosis on *Datura* similar to those given by Brownell X; + + + = more severe symptoms on *Datura* than those given by Brownell X.

gave no apparent reaction at the second inoculation, the seedlings reacted necrotically in both series, although six of them developed systemic necrosis at the November inoculation.

The results in Table 3 after two *Datura* passages to stabilize the strains, show that six of the seedlings reacted in the same way with respect to the X complex in both series, four giving an X similar to Brownell and two giving masked types. Of the others, three gave a masked strain at the November inoculation and a Brownell type X at the December inoculation, whereas the other three gave the opposite result to this. The only strain which appeared more severe than Brownell X came from 50-4 in the November series. One of the necrotic strains obtained in the earlier experiments came from a seedling with severe systemic necrosis to X, but this is not always so, as the other necrotic strain came from a localized reactor.

The X inoculated plants of the 12 seedlings from both series in Table 3 were tested serologically with Brownell X antiserum. The results paralleled those obtained with *Datura* in that where *Datura* gave definite mottle symptoms a positive precipitin reaction was obtained, and where *Datura* was free from symptoms the corresponding potato seedling gave no precipitin reaction except with 50-58 in the November series.

It can be concluded from these results that it is relatively easy to effect a separation of X strains from a complex by using small numbers of seedlings. The results indicate that X sensitive reactors are necessary for strain separation, although in Table 3 two of the seedlings 50-12 and 50-58 which did not react necrotically in the December series developed masked strains. It is probable that the necrotic reaction of the selected seedlings eliminates the severe and milder strains and so allows the development of the masked strain. The attenuation produced in 50-12 and 50-58 in the second series indicates that potato seedlings can cause a separation through an intracellular reaction which is not visible externally. Johnson (1947) was able to attenuate tobacco mosaic virus in sea holly without the development of necrotic lesions.

VI. THE CHARACTERISTICS OF SOME OF THE MASKED STRAINS OF VIRUS X SEPARATED BY THE POTATO SEEDLING TECHNIQUE

Sixteen masked strains, seven from the later group of experiments cited in Table 3 and nine from the first group were transferred to *Datura* plants. The masked strains from the earlier experiments were obtained by inoculation to *Datura* from tubers of the respective seedlings. In all instances complete protection from a severe strain was afforded in these *Datura* plants.

The masked strains were inoculated from this series of *Datura* plants to duplicate plants of *Datura stramonium* and *D. metel* all of which were rather large for inoculation as they were commencing to flower. They grew vigorously and flowered but showed no X symptoms. Twenty-eight days after inoculation of the *D. stramonium* and *D. metel* plants with the masked strains, five of the *D. stramonium* plants, each with a different masked strain, were used to inoculate young plants of tobacco and *N. campanulata*. At the same time five severe

strains and Brownell X were inoculated to a separate batch of tobacco and *N. campanulata* plants for comparative purposes. After these inoculations the *D. stramonium* and *D. metel* plants carrying the masked strains were reinoculated with a severe strain of X.

The results are given in Tables 4 and 5.

Table 4 shows that only four of the masked strains, those from 26-22, 31-45, 35-133, and 50-57D caused complete protection from the severe strain in the maturing *D. stramonium* and *D. metel* plants. Eight of the masked strains provided no protection in the plants of either species. The other four strains from 15-2, 30-103, 30-115, and 50-12D resulted in almost complete protection in *D. stramonium* and practically no protection in *D. metel*. In this latter group 30-103 and 30-115 contained mild rather than masked strains. Lack of complete protection by masked strains in potato has already been hinted at by Bald and White (1942).

TABLE 4

EFFECT OF INOCULATING *D. STRAMONIUM* AND *D. METEL* PLANTS WITH A SEVERE STRAIN OF X,
28 DAYS AFTER PREVIOUS INOCULATION WITH MASKED STRAINS

Potato Seedling from which Masked X obtained	Reactions on Duplicate Plants of <i>D. stramonium</i>		Reactions on Duplicate Plants of <i>D. metel</i>	
15-2	O	O	+ + +	+ + +
21-5	+ + +	+ + +	+ + +	+ + +
25-178	+ + +	+ + +	+ + +	+ + +
26-22	O	O	O	O
30-18	+ + +	+ + +	+ + +	+ + +
30-103	+	+	+ + +	+
30-115	+	+	+ + +	+ + +
31-45	O	O	O	O
35-133	O	O	O	O
50-10N	+ + +	+ + +	+ + +	+ + +
50-12D	O	+ + +	+ + +	+ + +
50-56N	+ + +	+ + +	+ + +	+ + +
50-57N	+ + +	+ + +	+ + +	+ + +
50-57D	O	O	O	O
50-58D	+ + +	+ + +	+ + +	+ + +
50-59D	+ + +	+ + +	+ + +	+ + +

Note.—O = no symptoms on *Datura stramonium* or *D. metel* after reinoculation with severe X; + = very mild mottle; + + + = very severe mottle and necrosis.

These results indicate that there are two types of masked X. The first type is able to maintain a sufficient concentration in the host tissues for protection to a severe strain at all growth stages of the plant. The other masked X is apparently able to build up in sufficient concentration for protection in young actively growing plants, but as the host plant ages, this strain diminishes in concentration and so exposes loci for the attachment and multiplication of severe strains. The rate at which the concentration of this latter type of masked strain diminishes is also dependent on the kind of host plant, as evidenced by the results with *D. stramonium* and *D. metel* in Table 4.

Table 5 also supports the possibility that there are two types of masked X. The strains from 15-2, 21-5, 25-178, 30-18, and 50-57D were obtained from the *D. stramonium* plants of Table 4 before these plants were reinoculated with severe X. In the experiments shown in Table 4, the strains from 21-5, 25-178, and 30-18 were not able to afford protection from a severe strain, whereas in the experiments of Table 5 these strains were able to build up in sufficient concentration in the young *N. campanulata* and tobacco plants to give protection. In addition, Table 5 shows that under the winter conditions existing the masked strains developed mild symptoms in some of the plants of the two species, particularly in tobacco. The symptoms consisted of a mild mottle with an occasional light white ringspot.

TABLE 5

RESULTS OF INOCULATING MASKED X STRAINS FROM OLD *D. STRAMONIUM* PLANTS TO TWO *NICOTIANA* SPECIES

All the results were obtained 21 days after the *N. campanulata* and tobacco plants carrying the X strains from 15-2, 21-5, 25-178, 30-18, and 50-57D were reinoculated with severe X.

Masked X Strains from Old <i>D. stramonium</i> Plants	<i>N. campanulata</i>		<i>N. tabacum</i> <i>v. atropurpureum</i>		<i>N. tabacum</i> <i>v. atropur-</i> <i>pureum</i> + Mild Y Strain
	Symptoms	Precipitin Reaction	Symptoms	Precipitin Reaction	
25-144	+	+	+	+	+
26-79	+	+	+	+	+
30-113	+	+	+	+	+
50-4N	+	+	+	+	+
50-12N	+	+	+	+	+
Brownell	+	+	+	+	+
15-2	+	+	+	+	+
21-5	O	O	+	O	O
25-178	O	+	+	+	O
30-18	O	O	+	O	+
50-57D	+	+	+	+	O

Note.—The symptoms in the indicator plants are compared with those given by Brownell X as in Tables 3 and 4. With the precipitin reactions O = no precipitation; + = a light precipitation; + + = a medium precipitation; and + + + = a heavy precipitation.

Table 5 in conjunction with Table 4 emphasizes the basic differences between severe and masked strains of X. Masked strains like those from 15-2 and 50-57D are able to maintain a sufficient concentration for protection in a suitable host under most conditions. On the other hand, strains like those from 21-5, 25-178, and 30-18 need the young actively growing tissues of a favourable host for multiplication and the maintenance of a concentration affording protection from a severe strain. It is apparent from these studies that the term masked X should be confined to the reactions in *Datura stramonium*, as under certain conditions as in mid-winter, tobacco and other *Nicotiana* species will at times give a mild mottle with X strains that are masked in *Datura*. In contrast to the masked strains in Table 5, the severe strains give well-defined symptoms of varying degrees of severity in the different host plants. With the addition of

a mild strain of virus Y to tobacco, the symptoms produced by severe strains of X and Brownell X are accentuated whereas with the masked strains, accentuation of symptoms only occurred with 15-2X. The severest reactions shown in Table 5 resulting from the combination with a mild strain of virus Y in tobacco were intensely necrotic, resulting in the death of some leaves and necrotic streaking of the stems.

In Table 5 the precipitin reactions shown are light or absent with masked strains, and are heavy in severe strains. Where no reaction was obtained, the tubes containing the reactants were held up to five hours at 37°C. without result. In these cases also, antiserum dilutions of 1:10, 1:20, and 1:50 were tried, and these gave no precipitin reaction with the plant extracts. These results support the general contention that the properties of masked compared with severe strains are due to the fact that masked strains are only able to develop in low concentration in host plants whereas severe strains maintain a high concentration.

VII. DISCUSSION

The process of strain separation described in this paper is apparently due to the selected potato seedlings having a less complete necrotic reaction to all the components of a virus X complex than is found in the variety Epicure. In some instances, all but the masked strains are localized, while in two seedlings a severe systemic necrosis resulted in the development of necrotic strains, probably through the tissues encouraging the development of the necrotic strain in high concentration to the exclusion of the other strains. The other necrotic strain came from a localized reactor, so the mechanism involved is not clearly defined. That the process is influenced by environmental conditions is shown by the fact that localization can be complete under midsummer conditions, whereas in mid-winter most seedlings are ineffective as strain separators. The evidence suggests that some of the selected seedlings are more efficient than others with respect to strain separation, but such differences tend to be obscured by the effects of external conditions.

The possibility cannot be overlooked that the necrotic reaction may not be necessary to strain separation. The process Johnson (1947) describes for the tobacco mosaic virus is apparently due to the intracellular encouragement of one strain and the suppression of others, a reaction which could be basically similar to the one described in this paper.

All the X strains which have been produced in these experiments are not necessarily pure, although serial passages through *Datura* have shown a high percentage of them to be stabilized with respect to their symptoms in this and other hosts. The possibility that attenuated strains vary in their ability to maintain in aging host plants a concentration sufficient for protection against a severe strain is an interesting one. The protein metabolism of aging plants is not at such a high level as in young (Walkley and Petrie 1941), so that some attenuated strains, owing to their lack of biochemical vigour, are unable fully to parasitize their host plants, thus exposing points of attachment which can still be used by the more vigorous severe strains.

The separation of strains by potato seedlings may explain the origin of masked strains which have been found in cultivated varieties. It also provides an explanation for the occurrence of new and virulent strains of X (Larson 1943, 1947) in relatively new potato varieties which are susceptible to virus X. New potato varieties usually start field production free from virus X, even though they are X susceptible, and so it is quite possible for them to develop apparently new strains of this virus when they become exposed to infection under the right conditions. This is an added point in favour of the view that all new varieties of potato developed by plant breeders should at least possess field immunity to virus X. These results also show that care needs to be exercised in testing progenies for resistance to virus X by the hand inoculation technique. In one experiment with 229 seedlings from a cross with the X immune seedling 41956 as one parent, 17.6 per cent. of the seedlings gave a necrotic reaction, and 25 of these carried a masked strain of X sufficient to give protection from a severe strain in subsequent inoculations. If testing were inadequate, and the reactions following the first inoculation of such seedlings with virus X were missed, the resultant protection due to the development of a masked strain could be confused with immunity to virus X.

VIII. ACKNOWLEDGMENTS

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IX. REFERENCES

- BALD, J. G. (1943).—Potato virus X: Mixtures of strains and the leaf area and yield of infected potatoes. *Coun. Sci. Industr. Res. Aust. Bull.* No. 165.
- BALD, J. G., and WHITE, N. H. (1942).—Potato virus X: The average severity of strain mixtures in three varieties of potato. *J. Coun. Sci. Industr. Res. Aust.* **15**: 300-6.
- BAWDEN, F. C., and ROBERTS, F. M. (1947).—The influence of light intensity on the susceptibility of plants to certain viruses. *Ann. Appl. Biol.* **34**: 286-96.
- BAWDEN, F. C., and SHEFFIELD, F. M. L. (1944).—The relationships of some viruses causing necrotic diseases of the potato. *Ibid.* **31**: 33-40.
- CADMAN, C. H. (1942).—Autotetraploid inheritance in the potato: some new evidence. *J. Genet.* **44**: 33-52.
- CLINCH, PHYLLIS E. M. (1944).—Observations on a severe strain of potato virus X. *Sci. Proc. R. Dublin Soc.* **23**: 273-99.
- COCKERHAM, G. (1943).—The reactions of potato varieties to viruses X, A, B, and C. *Ann. Appl. Biol.* **30**: 338-44.
- JOHNSON, J. (1947).—Virus attenuation and the separation of strains by specific hosts. *Phytopathology* **37**: 822-37.
- LARSON, R. H. (1943).—A foliar mottle and necrosis in Chippewa potatoes associated with infection by a strain of the potato X virus. *Ibid.* **33**: 1216-7.
- LARSON, R. H. (1947).—A mosaic disease of Mohawk potato caused by a virulent strain of the latent mottle virus. *Ibid.* **37**: 13.
- SALAMAN, R. N. (1933).—Protective inoculation against a plant virus. *Nature* **131**: 468.

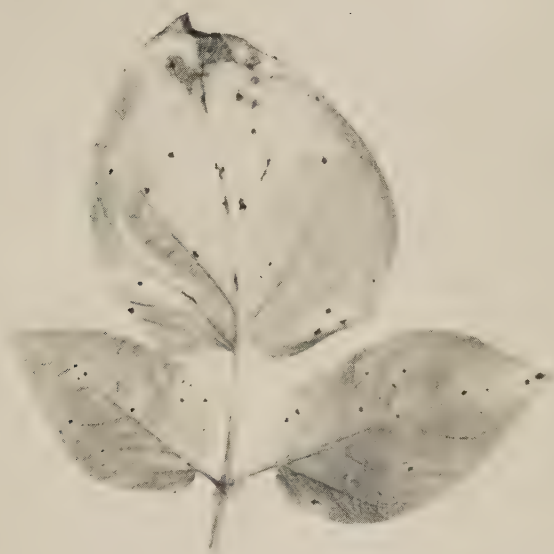


Fig. 1



Fig. 2

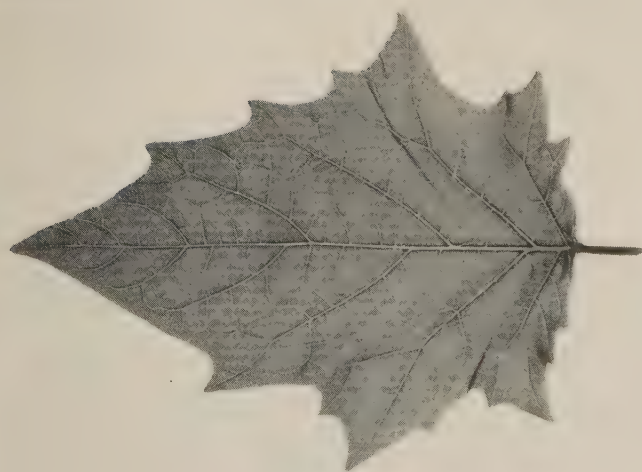


Fig. 1

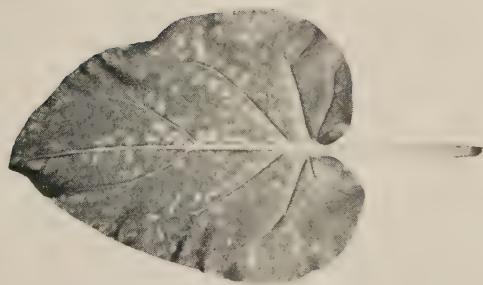


Fig. 2

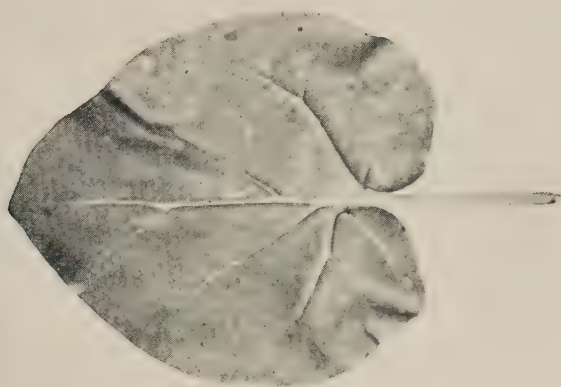


Fig. 3

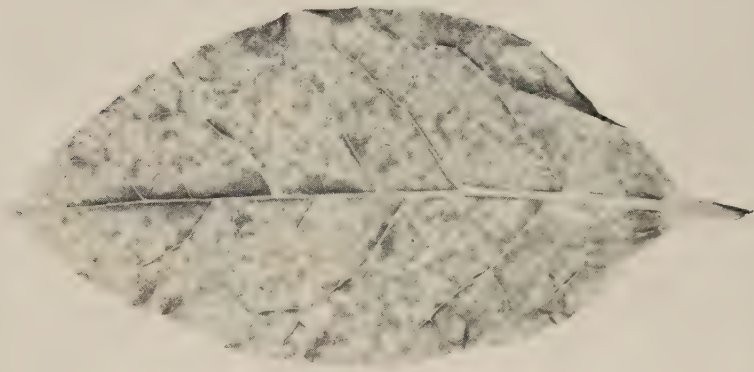


Fig. 3

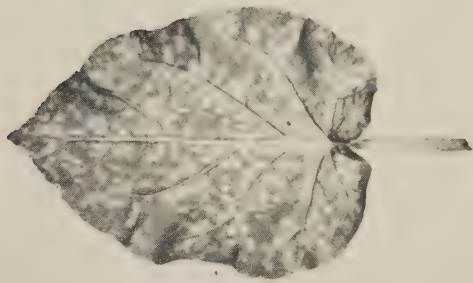


Fig. 2

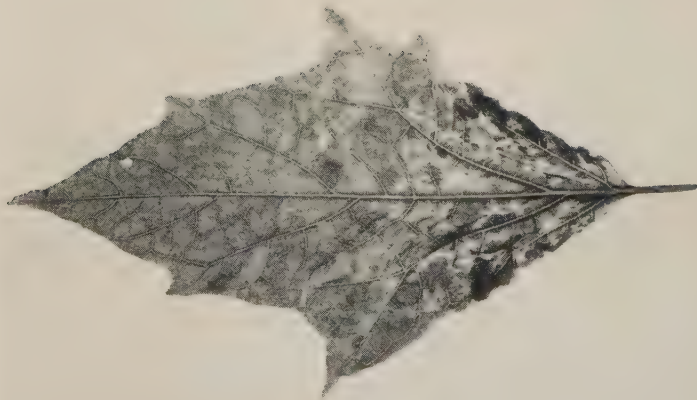


Fig. 1



Fig. 1



Fig. 2

HUTTON.—THE SEPARATION OF STRAINS FROM A VIRUS X COMPLEX BY PASSAGE THROUGH
POTATO SEEDLINGS

- SALAMAN, R. N. (1938).—The potato virus "X"; its strains and reactions. *Philos. Trans.* B 229: 137-217.
- WALKLEY, JOAN, and PETRIE, A. H. K. (1941).—Studies on the nitrogen metabolism of plants. IV. On the changing nature of the relation between proteins and amino-acids. *Ann. Bot.* 5: 661-73.

EXPLANATION OF PLATES 1-4

PLATE 1

Figs. 1, 2.—Necrotic reactions on the leaves from two different potato seedlings following inoculation with the Brownell X complex.

PLATE 2

- Fig. 1.—*Datura stramonium* leaf containing masked X.
- Fig. 2.—*Nicotiana glutinosa* leaf containing Brownell X.
- Fig. 3.—*N. glutinosa* leaf containing masked X.

PLATE 3

- Fig. 1.—*D. stramonium* leaf containing necrotic X.
- Fig. 2.—*N. glutinosa* leaf containing necrotic X.
- Fig. 3.—*N. tabacum* leaf containing necrotic X.

PLATE 4

- Fig. 1.—The effect of necrotic X on a plant of the Sebago potato.
- Fig. 2.—The effect of necrotic X on a plant of the Katahdin potato.

THE STRUCTURE OF PLASTIDS AND OTHER CYTOPLASMIC BODIES IN FIXED PREPARATIONS OF EPIDERMAL STRIPS

By J. G. BALD*

(Plate 1)

[*Manuscript received October 6, 1948*]

Summary

The fixation of the stromatic structure of plastids was found possible by the use of mixtures designed for the fixation of viruses in infected plant tissues.

Other features of plastids seen in fixed material are described.

Bodies formerly assumed to be protein crystals are fixed in a form that suggests a less simple structure and possibly a more important function than that of reserve protein.

At times there seems to be an association between plastids and bodies that is partly dependent on incident light.

I. INTRODUCTION

The structure of plastids has been discovered mainly from observations on living material (Weier 1938; Jungers and Doutreligne 1943). Most of the established fixatives seriously distort the stroma, and in doing so they destroy the plastids' most characteristic structural feature (Zirkle 1926). During experiments with fixatives intended to facilitate the staining of viruses in infected plant tissues (Bald 1948*b*), it was found that the stromatic structure of the plastids was sometimes preserved. Fixatives were developed that consistently preserve this and possibly other essential features of the plastids.

In addition, granules that have been in one of their forms called by virus workers "cuboidal bodies" (Rawlins and Johnson 1925; Goldstein 1926; Holmes 1928; Clinch 1932; Woods 1933) have appeared as portions of composite structures that superficially were somewhat like immature plastids. If the whole structures have not previously been observed and described, it is because portions of them are artefacts due to these newly-developed fixatives; or else the more delicate parts are easily destroyed by other types of fixation. At present, the latter explanation seems more likely, because the frequency with which the whole structures are seen in fixed preparations increases as the accuracy with which the details of plastid architecture are fixed. The images of plastids and the so-called "cuboidal bodies" as they appear after fixation will be briefly described.

II. MATERIALS AND METHODS

The test material used in the development of fixing and staining methods for viruses (Bald 1948*a*) was largely epidermal strips from tobacco and occasionally from other plants. Generally some parenchymatous cells were stripped off with the epidermis, and the fully developed chloroplasts they contained were available

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for comparison with the smaller plastids of the epidermis. The descriptions and illustrations in this paper are taken from healthy control tobacco plants.

Two fixatives will be described here. The first may be used for the preparation of material to demonstrate the stromatic structure of the plastid, the second to demonstrate also the structure of the cuboidal bodies. Further particulars of fixation and staining methods may be found elsewhere (Bald 1948*b*).

Fixative 1*b* may be made by mixing 50 ml. alcohol, 20 ml. Lugol's iodine, and 30 ml. 1.5 per cent. chromic acid immediately before use. Lugol's iodine consists of 6 g. potassium iodide and 4 g. iodine in 100 ml. water. Fix epidermal strips 20 minutes or longer in the dark. Wash in several changes of 50 per cent. alcohol in the dark; or in 0.5 per cent. sodium thiosulphate in 50 per cent. alcohol if precipitates tend to form. Pass through graded alcohols to 95 per cent., and leave 1 hour or longer to harden before staining.

The other fixative is 3*a* plus 1 per cent. carminic acid (Bald 1948*b*). It is made with 50 per cent. alcohol, 40 per cent. glacial acetic acid, and 10 per cent. water by volume, 1 g. cerium nitrate and 1 g. carminic acid. If necessary, heat to dissolve the carminic acid; and filter if the mixture is not clear. Fix epidermal strips 15 minutes or longer, transfer through several changes of 95 per cent. alcohol, and after an hour or more pass through 85 per cent. to 70 per cent. alcohol. Leave overnight before staining. The carminic acid is not used as a stain but as a fixative. Possibly it has some mordanting action for trypan blue (McWhorter 1941; Bald 1948*b*), which, of the stains tested, was the one that best reveals the full structural details of the so-called "cuboidal bodies."

III. THE PLASTID

Figure 1 illustrates the structure of the plastid of the tobacco plant as it appears after fixation. It is semi-diagrammatic in that no staining schedule has yet been found to differentiate with such clarity in one preparation all the structural features shown in this figure. The stroma, properly stained, appears more or less clearly according to the size and condition of the plastid (Fig. 1 and Plate 1, Fig. 1). Large chloroplasts in tissues fixed early in the morning reveal the stroma most clearly. The stromatic structure is probably permanent, although it may be obscured in living plastids of plants submitted for some time to bright daylight (Weier 1938*b*). It is present in the smaller plastids of the epidermis and the larger chloroplasts of the mesophyll.

The stromatic structure is confined to an outer shell. In tobacco and several other plants examined, the peripheral continuity of the stroma seems often (possibly always) to be broken by at least one circular gap. More than one gap is sometimes found, but whether the extra gaps are normal or a result of fixation has not been determined. The presence of gaps in the stroma of living chloroplasts was deduced by Zirkle (1926). At the edge of this gap, or occasionally elsewhere on the plastid, is a single refractile deeply-staining granule, too small to be resolved except under the highest power of the light microscope. Although it cannot be seen on every plastid or in every preparation, heavy staining, particularly with Giemsa, and subsequent destaining to a light colour will reveal it in

the majority of plastids. Iron-alum haematoxylin, and other stains may also be used to demonstrate it. There is difficulty because of its small size in detecting the colour it may assume. It is sometimes clearly situated in a round lighter-coloured area larger than could be attributed to diffraction effects, but the clear space might be a result of fixation. The refractile granule can be seen on conveniently placed plastids, to be situated no deeper than the stromatic layer.

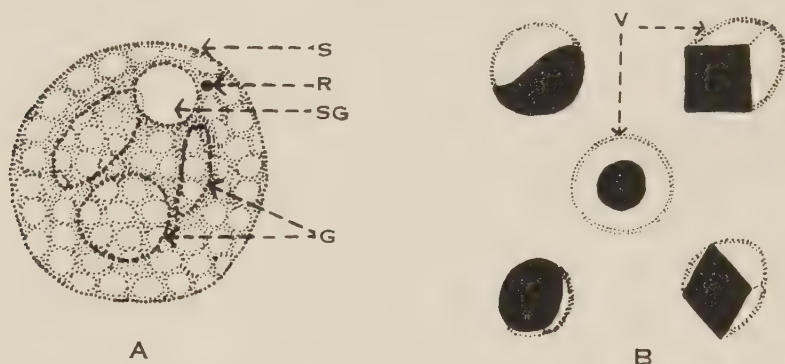


Fig. 1A.—Chloroplast from sub-epidermal mesophyll cell of tobacco leaf, showing stroma (S) containing starch grains (G) surrounded by cytoplasmic material, and penetrated by stromatic gap (SG) beside which is a minute refractile granule (R).

Fig. 1B.—Various forms of so-called “cuboidal bodies” at $\times 1\frac{1}{4}$ to $\times 2$ the magnification of the plastid. A structure like a delicate vesicle (V) is attached to each body.

The starch may be revealed by the haematoxylin iodine method (Bald 1948*b*) after 1b fixation. The starch grains do not always stain uniformly. Sometimes the centre is light coloured, and parts or all of the periphery darker. Seen at right angles to their broadest surface, they appear dark and rod- or slightly crescent-shaped. If they are fairly small compared with the size of the plastid that contains them, they may be grouped in a ring inside and around a somewhat enlarged stromatic gap.

Cytoplasmic material, in which the starch grains are embedded, appears to occupy the centre of the plastid, and is surrounded by the stroma. It may sometimes be seen even when the network of the stroma is definitely stained, and thus the starch grains may be more or less clearly outlined. When the plastids are gorged with starch the stroma may be barely visible, or invisible, and the material around the starch may outline the grains very clearly.

The presence of a containing semi-permeable membrane around the whole plastid has been deduced from the vesiculation of plastids under hypertonic conditions (Weier 1938*a*; Esau 1944). Zirkle (1926) claims to have shown the presence of a thin clear boundary layer outside the stroma. Occasionally during the present studies it has seemed that there was a layer outside the stroma of many plastids, sometimes more, sometimes less deeply stained than the stroma.

IV. CUBOIDAL OR SPHERICAL BODIES

The granules, of which the “cuboidal bodies” are one form, are preserved in part by the majority of fixatives, whether or not the plastids are well fixed or

the mitochondria are preserved. The granules accept a number of stains, but are most brilliantly coloured by the acid fuchsin of combinations containing it (Woods 1933). They are not always cuboidal in form; more often they are spherical, sometimes irregular, and they vary in size. They never appear to be as small as spherical mitochondria. Many are much larger than the 0.8 micron given by Woods as a maximum measurement. They are free in the cytoplasm or adhere to plastids. They may be observed in the streaming cytoplasm of living hair cells or in the elongated epidermal cells from beneath the veins, if the cells are stained *in vivo* with dilute Janus green according to the method given by Sorokin (1941).

As methods of fixation employed in the virus studies (Bald 1948*b*) became reasonably adequate, some images were obtained that suggested variations in staining within a single spherical or cuboidal body. Often a clear area showed around a deeply staining cuboid or sphere or the central part was clearer than the outer part. Finally, preparations were obtained in which the walls of what might be interpreted as a fine vesicle were stained (Fig. 1*B* and Plate 1, Fig. 2). This appeared surrounding or at one side of the deeply staining granule. When the granule was spherical, the image was somewhat like that occasionally given by immature plastids. The vesicle was best preserved and stained by fixative 3a plus 1 per cent. carminic acid, followed by trypan blue and orange G (Bald 1948*b*). Other stains, including iron-alum haematoxylin sometimes differentiated the fine vesicle wall. In some preparations a considerable proportion of granules appeared to have vesicles attached, while others had no sign of them. Some of the forms they took are shown in Figure 1*B*. They included, besides, vesicles surrounding only a dot of deeply staining material, and apparent division figures.

In some preparations these bodies were plentiful in the cytoplasm. No more adhered to plastids than might have been expected from random contact during streaming and adhesion until the cytoplasmic currents pulled them apart again. In other preparations many more of the bodies adhered to plastids than would be expected from random contact (Plate 1, Fig. 1). In others again there seemed to be fewer bodies in the cells. Examination of plastids in some of these preparations often revealed images that might be interpreted as indicating a release of the stainable material of the granule into the plastid, or collection of stainable material in the plastid to form a granule.

It would be possible to illustrate these appearances as a sequence, and suggest a functional association between plastids and bodies. However, the adequacy for this purpose of the fixing and staining techniques would have to be examined more carefully, and critical experiments would be needed to test such an interpretation. Following is the principal evidence so far accumulated that might be interpreted as suggesting an association.

In epidermal strips from leaves of healthy plants collected in the greenhouse about midday or early in the afternoon of bright sunny days, the bodies were mainly distributed throughout the cytoplasm. In strips from leaves collected at 8 or 9 a.m. on dull mornings, there were many examples of adherence, and of the presence of material in the plastids that stained in the same way as the cuboidal or spherical bodies.

Leaves from healthy tobacco plants were collected about 3 p.m. on sunny days, and split into two pieces down the midrib. Epidermal strips were taken from half of each leaf and fixed immediately. The other half leaves were kept damp in a dark cupboard for 2 hours before strips were taken from them and fixed. In the epidermal strips from half leaves kept in the dark, there appeared to be more frequent association of plastids and bodies than in those submitted to bright light.

A phenomenon that might lead to the alternative explanation, that these appearances are, at least in part, artefacts of fixation, is described by Dufrenoy, Stamatinis, and Sarejanni (1929). They describe a dissociation of protein and lipid material in diseased plastids during fixation with Nemec's and other mixtures. Some of the mixtures evolved for the fixation of viruses (Bald 1948*b*) produced similar effects in healthy plastids. It is possible that the best fixation so far attained may fail to prevent this dissociation, and many of the images indicating association of plastids and protein or cuboidal bodies may have been due to poor fixation of the living structure. Only further critical work can decide the point.

V. REFERENCES

- BALD, J. G. (1948*a*).—A method for the selective staining of viruses in infected plant tissues. *Phytopathology Amer. J. Bot.* (in press).
- BALD, J. G. (1948*b*).—Additional methods for fixing and staining viruses in infected plant tissues. (In press.)
- CLINCH, PHYLLIS (1932).—Cytological studies of potato plants affected with certain virus diseases. *Sci. Proc. R. Dublin Soc.* **20**: 143-72.
- DUFRENOY, J., STAMATINIS, N., and SAREJANNI, J. (1929).—Études cytologiques sur la mosaïque du tabac. *Rev. Path. Vég.* **16**: 106-17.
- ESAU, KATHERINE (1944).—Anatomical and cytological studies on beet mosaic. *J. Agric. Res.* **69**: 95-117.
- GOLDSTEIN, BESSIE (1926).—A cytological study of the leaves and growing points of healthy and mosaic diseased tobacco plants. *Bull. Torrey Bot. Cl.* **53**: 499-599.
- HOLMES, F. O. (1928).—Cytological study of the intracellular body characteristic of Hippeastrum mosaic. *Bot. Gaz.* **86**: 50-8.
- JUNCERS, V., and DOUTRELIGNE, J. (1943).—Sur la localisation de la chlorophylle dans les chloroplastes. *Cellule* **49**: 409-17.
- MCWHORTER, F. P. (1941).—Isometric crystals produced by Pisum virus 2 and Phaseolus virus 2. *Phytopathology* **31**: 760-1.
- RAWLINS, T. E., and JOHNSON, J. (1925).—Cytological studies of the mosaic disease of tobacco. *Amer. J. Bot.* **12**: 19-32.
- SOROKIN, HELEN (1941).—The distinction between mitochondria and plastids in living epidermal cells. *Ibid.* **28**: 476-85.
- WEIER, E. (1938*a*).—The structure of the chloroplast. *Bot. Rev.* **4**: 497-530.
- WEIER, E. (1938*b*).—Viability of cells containing chloroplasts with an optically homogeneous or granular structure. *Protoplasma* **31**: 346-50.
- WOODS, M. W. (1933).—Intracellular bodies associated with ring-spot. *Contr. Boyce Thompson Inst.* **5**: 419-34.
- ZIRKLE, C. (1926*a*).—The structure of the chloroplast in certain higher plants. I. *Amer. J. Bot.* **13**: 301-20.
- ZIRKLE, C. (1926*b*).—The structure of the chloroplast in certain higher plants. II. *Ibid.* **13**: 321-41.

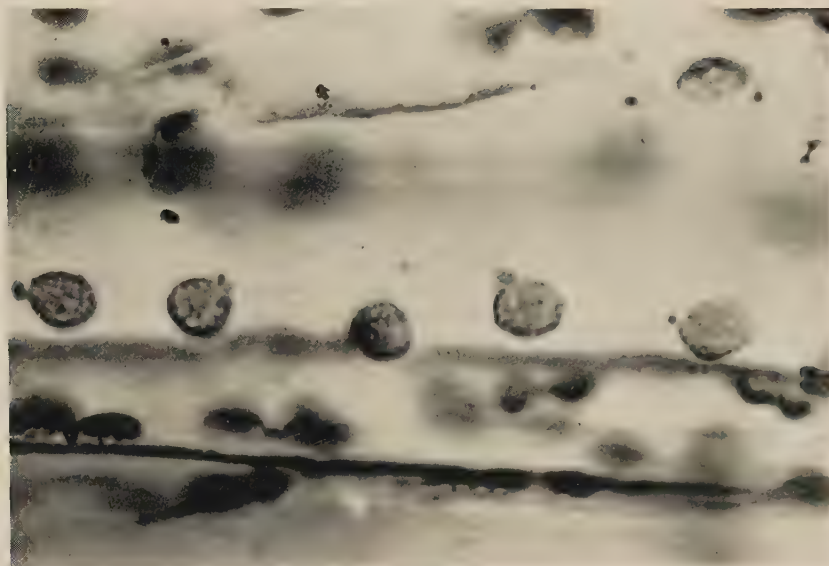


Fig. 1

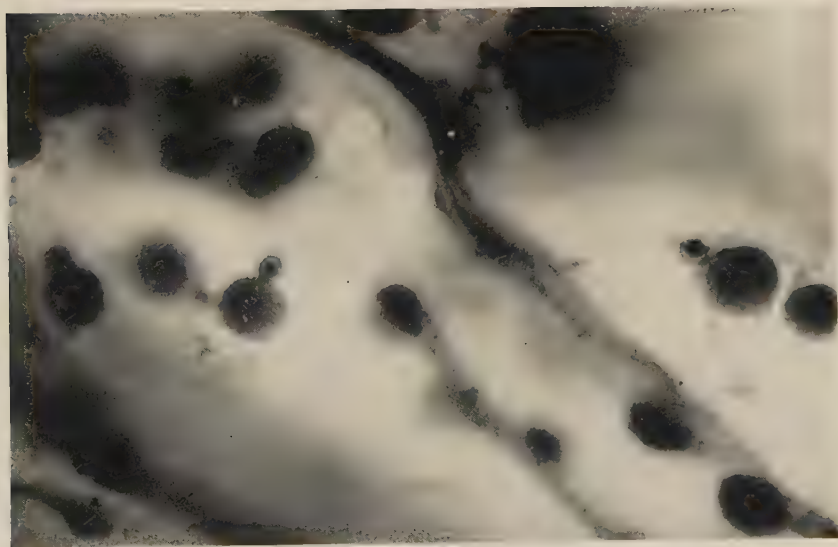


Fig. 2

BALD.— THE STRUCTURE OF PLASTIDS AND OTHER CYTOPLASMIC BODIES IN FIXED PREPARATIONS
OF EPIDERMAL STRIPS

EXPLANATION OF PLATE 1

Fig. 1.—A row of 5 plastids showing fixation of stromatic structure. So-called “cuboidal bodies” adjacent to four of the plastids. Fixation 1b, stain Giemsa. *c.* x 850.

Fig. 2.—Two plastids with the spherical form of “cuboidal bodies” attached. The vesicular portion of these bodies is well fixed. The stromatic structure of the plastids is faintly evident, but the staining is too deep to demonstrate it clearly. Fixative 3a plus 1 per cent. carminic acid. Stain, trypan blue and orange G. *c.* x 850.

THE DEVELOPMENT OF AMOEBOID INCLUSION BODIES OF TOBACCO MOSAIC VIRUS

By J. G. BALD*

(Plates 1-2)

[*Manuscript received October 6, 1948*]

Summary

Fixatives that preserve the stromatic structure of normal plastids, show "amoeboid" inclusion bodies also to have a stromatic structure of a coarser and less regular type.

Plastids may be found with distortion of the stroma and an accompanying staining reaction like that given by virus inclusions.

A series of stages may be found between normal plastids and amoeboid inclusion bodies. The inclusion bodies appear to consist of one or more than one distorted and aggregated plastids.

The possibilities of staining methods for the study of virus-host cell relations is discussed.

I. INTRODUCTION

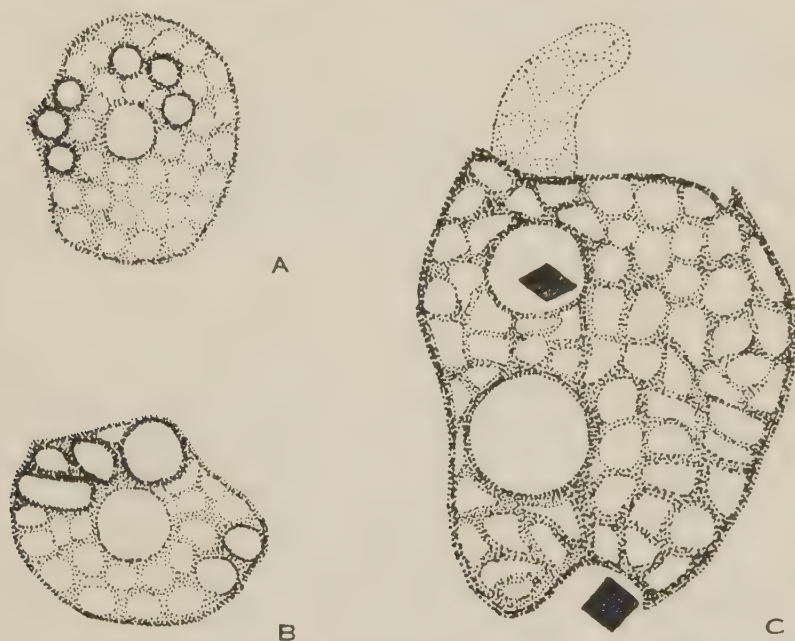
During work on the fixation and staining of viruses in infected plant tissues (Bald 1948*a*) some evidence was obtained that amoeboid inclusion bodies may develop from plastids. The only type of plant virus inclusion body whose development has previously been studied in detail, is the granular inclusion of aucuba mosaic of tomato (Sheffield 1931). This virus is a strain of ordinary tobacco mosaic. The granular inclusions are formed by aggregation of visible granules presumably developing in the infected cells, and carried passively in the streaming cytoplasm. The granular inclusions and the crystalline inclusions of tobacco mosaic were later shown (Sheffield 1939) to contain virus in an infective form. The inclusions were dissected out of infected cells, and were used to produce infections by inoculation to healthy plants. This work was repeated with the amorphous inclusion bodies of severe etch virus (Sheffield 1941). Thus some typical inclusion bodies contain a high concentration of virus, and material derived from infected cells (Bawden 1943).

The structure of plastids as it appears after fixation in mixtures that preserve their stromatic character has been described in another paper (Bald 1948*b*). Apart from the stroma, the main features of the fixation image are (i) a region not covered by the stroma, that has been called, for convenience, the stromatic gap, (ii) the starch grains embedded in cytoplasmic material inside the stroma, and (iii) outside the stroma, probably a semi-permeable membrane. A small refractile granule is often situated on the edge of the stromatic gap. These three features of the fixation image remain to be confirmed from living material, or material fixed by other methods.

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II. ABNORMAL PLASTIDS AND AMOEBOID INCLUSIONS

Plastids in epidermal cells of infected leaves, hardly distinguishable from normal plastids, are occasionally found to stain purple or blue with Giemsa, the colour characteristic of virus inclusions. It is more common, however, to find plastids in which the first accompaniment of this staining reaction is an enlarged stromatic gap, or some emphasis or distortion of the stromatic pattern (Figs. 1A, 1B). Apparently before this stage of development is reached, the starch has been converted to sugars, because only in rare instances has the haemotoxylin-iodine staining schedule (Bald 1948*a*) revealed even the remnants of starch grains in distorted plastids. Sometimes at this or a somewhat later stage a "cuboidal body" is seen in the centre of what is probably the enlarged stromatic gap, and occasionally a refractile granule on the edge of the gap. The body enlarges as



Figs. 1A and 1B.—Two abnormal plastids, sketched from camera lucida drawings and enlarged. Plastid (A) illustrates deeper staining and slight distension of 6 pores in the stroma. Plastid (B) illustrates deeper staining, definite enlargement, and distortion of stromatic structure. *c. x 4500.*

Fig. 1C.—An amoeboid inclusion body, rather odd in shape but otherwise typical. Two cuboidal bodies are associated with it. The protrusion above probably represents a distorted leucoplast; the main portion of the inclusion is probably composed of three or four misshapen and aggregated plastids. The original stromatic structure is distorted but preserved. *c. x 3600.*

the stromatic gap and some or all of the stomatal pores enlarge, and it begins to assume its mature form. If it is of a size that would suggest it arises from a small leucoplast in an epidermal cell, only the vacuole developing from the gap may be prominent; if it is of a size that would suggest it arises from a large chloroplast it is more likely to have the form of a multivacuolate network (Fig.

1C and Plate 1, Fig. 1) . According to the fixation and staining schedule adopted, the body now stains with Giemsa as a network the same colour as the crystalline inclusion, or half-way between this colour and that taken by the normal plastid. The intensity of colouration may vary to a degree that suggests variation in the concentration of virus.

The distorted plastids or small amoeboid bodies apparently tend to aggregate (Plate 1) and round off to form larger amoeboid bodies. In leaves of tobacco plants about 10 days after inoculation with mosaic, these aggregations are often present in every stage of development. Probably, plastids that remain apparently normal in infected cells for some time also can go through the same cycle, and develop into new amoeboid bodies; for stages intermediate between plastids and fully formed amoeboid bodies have been found in plants infected for a considerable time. Unless new plastids develop from existing primordia or mitochondria, this might contribute to an exhaustion of the chlorophyll and premature yellowing of the leaf.

In the fully developed amoeboid inclusion it is common to find a number of cuboidal bodies, or the spherical forms of them (Fig. 1 and Plate 1) in the centre of the larger vacuoles. This has been observed by a number of workers (Kunkel 1924; Rawlins and Johnson 1925; Goldstein 1926; Holmes 1928; Clinch 1932; Woods 1933). The frequency with which it occurs is often higher than the frequency with which plastids and bodies are associated in normal cells under conditions of bright daylight (Bald 1948*b*).

Following is an observation made several times on epidermal strips from plants on which symptoms had just appeared. Inclusion bodies were just beginning to form and the little material in the cytoplasm that gave the virus staining reaction was mainly still in diffuse form. In control material from healthy plants there was only rare association between plastids and spherical or cuboidal bodies. In infected strips there was more frequent association. In some cells of infected epidermal strips, particularly along the veins where virus was evident, nearly every plastid had around the stromatic gap, or in patches which showed very clearly on the grey-green plastids, material staining red with acid fuchsin like that in the granule of the cuboidal body (Plate 2). It is possible that the frequent association of plastids and cuboidal bodies had some association with virus multiplication, and was connected also with the red-staining masses in the plastids. Alternatively, the protein fractions of the plastids may have dissociated more easily in infected tissues than in healthy, aggregated within the plastids and stained with aniline-fuchsin (Dufrenoy, Stamatinis, and Sarejanni 1929).

III. DISCUSSION

These are preliminary observations, but they have been often repeated on material sampled over a period of more than one year. Some of the fixation and staining methods employed (Bald 1948*b*) both preserve the stromatic character of plastids, and preserve and facilitate the staining of virus. They are therefore more likely to reveal such a relationship between plastids and amoeboid inclusion bodies, if it exists, than established fixatives.

These results suggest many interesting speculations about virus multiplication and virus-cell relationships. The fixing and staining techniques described offer contributory methods for establishing or disproving them. Some evaluation of what these methods will and will not do therefore seems desirable.

The uses and limitations of the staining technique depend fundamentally on the manner and sites of virus multiplication. If viruses multiply by division of previous virus particles floating at random in the cell, there is little point in a detailed cytological study by these special methods of virus-cell relationships. Two other alternatives exist; that multiplication occurs by some such process as division, but mostly at certain points where suitable concentrations of metabolites and suitable energy conditions exist; or that, in order to reproduce, the virus particles become part of the structure of the cell, e.g. join with cell constituents to become one of the phospholipin-ribonucleoprotein complexes that are sometimes represented (Davidson 1945) as the characteristic self-perpetuating units of living matter. The latter alternative best explains the varied and complicated phenomena of immunity and interaction between related viruses (Price 1940; Bawden 1943; Bawden and Kassanis 1945).

According to this view of virus multiplication, virus particles, as distinct from unformed elements or precursors of particles, would exist in an infected cell (*a*) in active form at relatively limited loci, (*b*) passively concentrated around the multiplication loci, (*c*) dispersed throughout the cell in an inert form, or (*d*) as masses of virus particles aggregated into more or less clearly defined inclusion bodies. The position of these bodies in the cell might bear little relation to the loci of multiplication.

Classes (*c*) and (*d*) would intergrade; also aggregates might include other elements than virus particles. Inclusion bodies might vary in composition from almost pure virus to the products of abnormal metabolism, containing relatively little virus.

If the loci of multiplication were inside organs of the plant cell, inclusions might consist of these organs in a degenerate condition containing virus in both forms (*a*) and (*b*). If the organs had become completely degenerate and their loci of virus multiplication were no longer active, the virus might be in form (*b*) only.

Although they are in the highest degree speculative, these assumptions are not unreasonable, and they put reasonable bounds on what results may be expected from staining techniques. Some of these are:

(1) It is possible that the small amounts of active virus at the multiplication loci would never be revealed by staining.

(2) Possibly, also, only viruses that multiply sufficiently in host cells to produce a great excess of virus particles above the number needed to fill active centres of multiplication are likely to be revealed by selective staining.

(3) It does not follow that the location of virus in high concentration automatically discovers the centres of multiplication. Virus particles probably circulate freely in the microscopically structureless portions of the cell cytoplasm

(Sheffield 1931), and must pass from cell to cell (Uppal 1934). It is possible even that virus particles may pass from cells where they developed to other cells before they aggregate into definite cell inclusions. However, the presence of legume virus inclusions in such *formed* elements as the nucleoli (McWhorter 1941), if these inclusions are finally proved to contain virus, might suggest that the virus multiplies in the nucleoli or at their borders. If confirmation is found for the hypothesis that the amoeboid inclusions of tobacco mosaic develop from plastids (see above), the presence of virus in these inclusions might suggest that virus multiplies in the plastids. *A priori*, there seems less chance of virus particles passing through a membrane or phase boundary and of being concentrated passively in an organelle in which they were not formed, than of virus particles concentrating in an organelle where they are being formed.

(4) It seems that the most likely organs for the multiplication of virus would be those, normally containing ribonucleic acid (Davidson 1945), in or near which high concentrations of virus often appear in infected cells. Great care would be needed in judging whether these concentrations of virus had not lodged at such points after circulation around the cell.

(5) In tissues beyond the meristematic stage, the organs of the cell that normally contain ribonucleic acid are the microsomes (if the microsomes in plant cells are analogous to the microsomes of animal cells), the mitochondria, the plastids, and the nucleoli. It would be interesting to discover if the so-called "cuboidal bodies" also contain ribonucleic acid. Special attention might be paid to the condition of these organs in healthy and diseased tissues.

IV. ACKNOWLEDGMENTS

The results contained in this and three preceding papers were obtained while I was working as a visitor in the Department of Plant Pathology, University of California, Berkeley, California. I wish to thank both C.S.I.R. for making this period of work possible, and members of the Plant Pathology staff, University of California, particularly Dr. M. W. Gardner and Dr. T. E. Rawlins, for laboratory accommodation, material, and help in many forms.

V. REFERENCES

- BALD, J. G. (1948*a*).—Additional methods for fixing and staining viruses in infected plant tissues. *Amer. J. Bot.* (in press).
- BALD, J. G. (1948*b*).—The structure of plastids and other bodies in fixed preparations of epidermal strips. *Aust. J. Sci. Res. B* 1: 452-57.
- BAWDEN, F. C. (1943).—"Plant Viruses and Virus Diseases." p. 290. (Chronica Botanica Co.: Waltham, Mass.)
- BAWDEN, F. C., and KASSANIS, B. (1945).—The suppression of one plant virus by another. *Ann. Appl. Biol.* 32: 52-7.
- CLINCH, PHYLLIS (1932).—Cytological studies of potato plants affected with certain virus diseases. *Sci. Proc. R. Dublin Soc.* 20: 143-72.
- DAVIDSON, J. N. (1945).—Cytoplasmic ribonucleoproteins. *Biochem. J.* 39: lix-lxi.

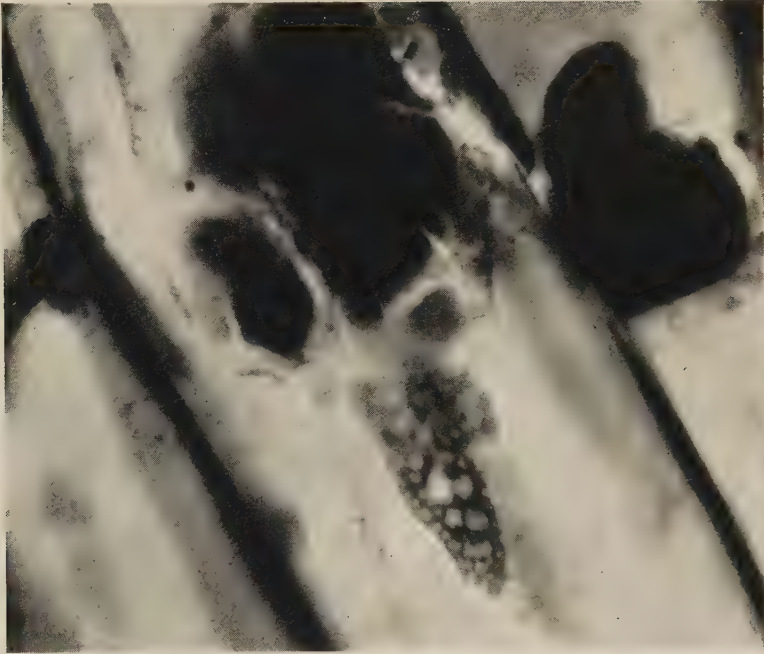


Fig. 1

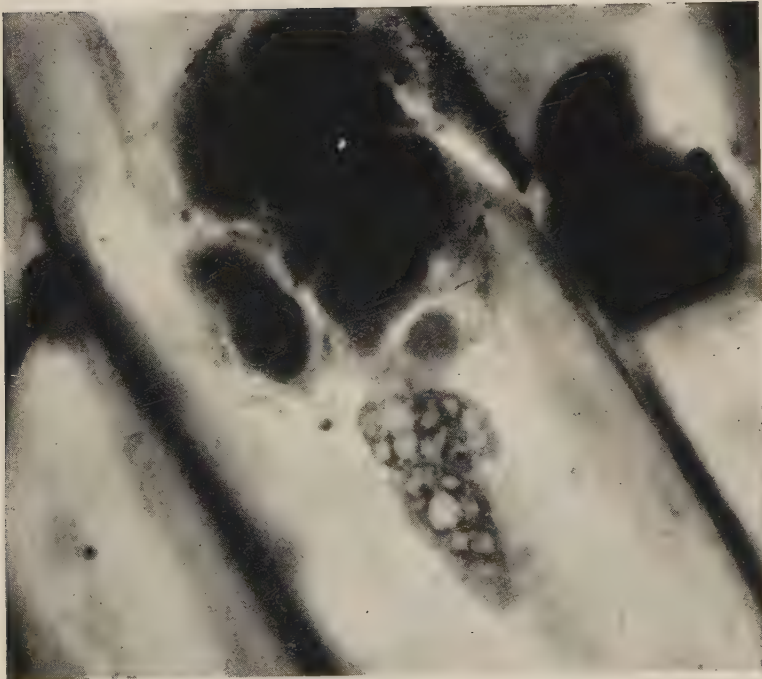


Fig. 2

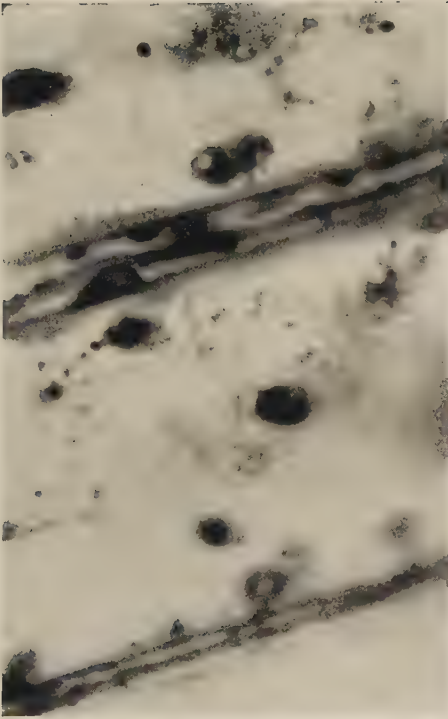


Fig. 1

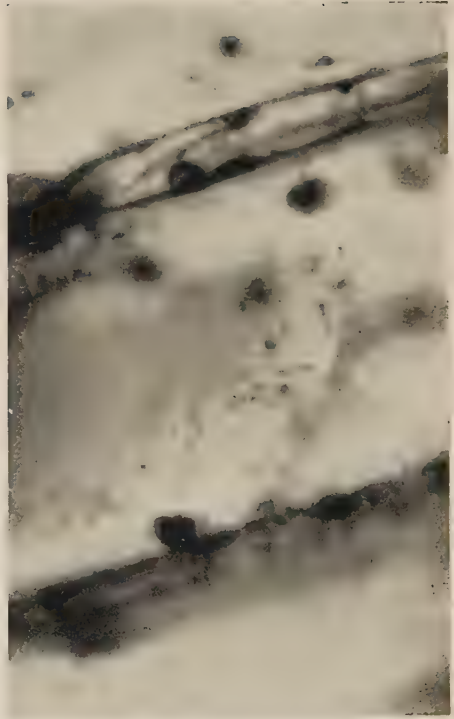


Fig. 2

BALD.—THE DEVELOPMENT OF AMOEBOID INCLUSION BODIES OF TOBACCO MOSAIC VIRUS

- DUFRENOY, J., STAMATINIS N., and SAREJANNI, J. (1929).—Études cytologiques sur la mosaïque du tabac. *Rev. Path. Vég.* **16**: 106-17.
- GOLDSTEIN, BESSIE (1926).—A cytological study of the leaves and growing points of healthy and mosaic diseased tobacco plants. *Bull. Torrey Bot. Cl.* **53**: 499-599.
- HOLMES, F. O. (1928).—Cytological study of the intracellular body characteristic of Hippeastrum mosaic. *Bot. Gaz.* **86**: 50-8.
- KUNKEL, L. O. (1924).—Histological and cytological studies on the Fiji disease of sugar cane. *Bull. Hawaii. Sug. Ass. Bot. Ser.* **3**: 99-107.
- MCWHORTER, F. P. (1941).—Isometric crystals produced by Pisum virus 2 and Phaseolus virus 2. *Phytopathology* **31**: 760-61.
- PRICE, W. C. (1940).—Acquired immunity from plant virus diseases. *Quart. Rev. Biol.* **15**: 338-61.
- RAWLINS, T. E., and JOHNSON, J. (1925).—Cytological studies of the mosaic disease of tobacco. *Amer. J. Bot.* **12**: 19-32.
- SHEFFIELD, F. M. L. (1931).—The formation of intracellular inclusions in Solanaceous hosts infected with aucuba mosaic of tomato. *Ann. Appl. Biol.* **18**: 471-93.
- SHEFFIELD, F. M. L. (1939).—Some effects of plant virus diseases on the cells of their hosts. *J. Roy. Micr. Soc.* **59**: 149-61.
- SHEFFIELD, F. M. L. (1941).—The cytoplasmic and nuclear inclusions associated with severe etch virus. *Ibid.* **61**: 30-45.
- UPPAL, B. N. (1934).—The movement of tobacco mosaic virus in leaves of *Nicotiana glauca*. *Indian J. Agric. Sci.* **4**: 865-73.
- WOODS, M. W. (1933).—Intracellular bodies associated with ring-spot. *Contr. Boyce Thompson Inst.* **5**: 419-34.

EXPLANATION OF PLATES 1-2

PLATE 1

Figures 1 and 2 are focused at different levels. Above are massive crystalline inclusions of tobacco mosaic, below an "amoeboid" inclusion body. Figure 1 shows clearly the stromatic structure of the amoeboid inclusion. Figure 2 shows that the inclusion is formed of two masses not yet completely fused. Four "cuboidal" bodies were visible in the whole body. One can be seen in Figure 1 and two in Figure 2, the fourth is out of focus at both levels. Fixative 1b plus carminic acid, stain safranin and Giemsa. *c.* x 850.

PLATE 2

Figures 1 and 2 show material staining red with acid fuchsin, in the same way as the granules of cuboidal bodies, clearly visible in leucoplasts. Epidermal cells from the underside of a vein of a recently infected tobacco leaf. The aggregation of virus in stainable quantities is only just beginning in adjacent cells. Fixation 3b, stain aniline-fuchsin Giemsa aurantia. *c.* x 850.

TEMPERATURE STUDIES OF THE HABITAT OF *EUTERMES EXITIOSUS* WITH SPECIAL REFERENCE TO THE TEMPERATURES WITHIN THE MOUND*

By F. G. HOLDAWAY† and F. J. GAY‡

(Plate 1)

[Manuscript received July 7, 1948]

Summary

A study was made to determine whether or not *Eutermes exitiosus* maintains within its mound a constant temperature at which it would be desirable to maintain artificial laboratory colonies of this termite.

It was found that the temperature of the mound is not constant. The temperature of a given portion of the mound varies with the time of day, and varies from day to day with changes in environmental temperature.

The temperature of the nursery exhibits less variation than other portions of the mound, but it is continuously higher than the temperature of the air or the soil, or of that portion of the mound which receives the greatest amount of heat from the sun. The temperature of the nursery follows a seasonal trend which roughly parallels the seasonal change in air temperature.

Although the temperature of a mound is not constant, and although it is related to the surrounding air temperature, it is affected by the presence of living termites in the mound, the temperature of an occupied mound averaging from 14.5° to 18.6°F. higher than it would if it were unoccupied. The temperature of an occupied mound is apparently maintained above that of an unoccupied mound by the metabolism of the termites. The number of individuals present in the mound in the summer is less than the number present in the winter. By virtue of the higher temperature of the mound in the summer, and the resultant higher metabolism of the termites, the smaller number of individuals present in the mound is capable of maintaining the mound temperature as much above that of an unoccupied mound as is the larger number present in the winter.

The presence of alates in the mound results in the temperature being from 10° to 13°F. higher than that in mounds of comparable shape and size in which there are no alates.

Termites, probably as a result of movement into the mound from galleries away from the mound, are capable of buffering the effect of sudden falls in air temperature.

The practical applications of the observations on mound temperature are as follows:

- (a) By recording mound temperature, it is possible to distinguish populous from non-populous mounds. This ensures that when a mound is selected to provide termites for laboratory colonies, the maximum number of termites procurable from a mound at that particular time of the year will be secured.

* Publication of this manuscript has been unavoidably delayed by several factors, the more important of which have been the departure of the senior author for Honolulu and the intervention of the war.

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- (b) By recording the temperatures of mounds chosen for field studies on the resistance of timbers and timber treatments, one can be sure of using only vital colonies, and can also keep a check on the vitality of the respective colonies throughout the period of the test.
- (c) By recording the temperature of mounds used for insecticidal studies, it is possible to compare the effects of various toxic materials on termite colonies which are known to be normally healthy at the time of treatment.

I. INTRODUCTION

Eutermes exitiosus Hill* is a species of termite which is responsible for considerable economic damage in the southern part of the Australian continent (Hill 1932). It has been utilized in investigations of the resistance of untreated and treated timbers and other materials to termite attack; and following the discovery that it could be kept in jars in the laboratory (Hill 1930) it has been selected for use in laboratory colonies for evaluating resistance under standard conditions (Holdaway 1935).

Many entomologists have thought that termites, like some other social insects, possess powers of regulating the conditions of temperature and humidity under which they live. Snyder (1929) has stated that the termite in whose mounds the Nile Monitor, *Varanus niloticus*, lays its eggs, maintains the nest at a constant temperature and humidity. Emerson (1937), also, has referred to the constant temperature and humidity of termites' nests. If *Eutermes exitiosus* could and did maintain a constant temperature in the nest, it was obviously desirable to determine what the temperature was, in order that it could be reproduced in the laboratory colonies.

Observations on the temperature of termite mounds are not numerous. One of the earliest observers appears to have been Petch (1906). His observations led to the conclusion that fluctuations in temperature of the mound of *Termes redemanni* were less marked than fluctuations of the external temperature. However, he did not have records of soil temperature for comparison. Hegg (1922), in discussing mound temperatures, stressed the need for further and more detailed studies.

Probably the most detailed observations made to date are those of Cowles (1930) who made an interesting study of the temperature of the mounds of the grass-feeding *Nasutitermes trinerviformis* from the point of view of the situations in which the eggs of the Nile Monitor are hatched. He concluded that the temperature within the mounds is more equable than that of the external environment. But even Cowles' observations, although much more detailed than those of Petch, would have been much more valuable had he been able to secure records from other places in the same general habitat.

Observations on the temperature of the habitat of social Hymenoptera are more numerous than those on termites. The forms studied most are the bee, the mound-building ants, and the social wasps. Gates (1914), Phillips and

* Many termite taxonomists consider that the generic name *Eutermes* should be replaced by *Nasutitermes*. However, in view of the widespread use of the name *Eutermes* in Australian literature, it has been retained here.

Demuth (1914), and Zander (1917) have studied the temperature of bee clusters. Numerous observations have been made on mound ants, the most comprehensive of which are probably those of Andrews (1927) on *Formica exsectoides*, and the reviews of Steiner (1930) and Himmer (1932). Andrews concluded that, because the temperature of an unoccupied mound was higher than the temperature of the soil, "the temperature is due to the sun's rays and not appreciably to the heat given off by the ants nor to any fermentation of the relatively small amount of vegetable matter contained in the mounds." Steiner gives a good summary of the evidence regarding the factors affecting the heat of the nests of the social Hymenoptera. Himmer concluded that the nature of the temperature regulation in the social Hymenoptera varies with the construction of the nest and with the capacity for heat production of individuals. It is best developed in the honey bee in which there is both chemical and physical heat regulation. The temperature in ants' nests is characterized by considerable dependence on outside influences according to the size and architecture of the nests: heat accumulation is more or less efficient and lasting, whilst chemical heat production may be marked or insignificant.

The primary aim of the studies here reported was to gain information on the temperatures under which *Eutermes exitiosus* lives. When it was discovered that a constant temperature was not maintained in the mounds, the experiments were extended with the following objects. Firstly, to study the temperature of a mound at all seasons of the year. Secondly, to determine whether the mound temperature is related to external environmental conditions, by comparing the temperature records obtained within a mound with those in the adjacent soil and that of the air. Thirdly, to determine the extent to which the temperature of a mound is influenced by the presence in it of living termites, by recording the temperatures of two similar mounds, killing the termites in one, and continuing to record the temperatures of both.

To obtain the data required, it was found necessary to use, in all, four pairs of mounds. These, which were all situated on a forest-covered hill within a mile or so of the laboratories of the Division of Economic Entomology, C.S.I.R., at Canberra, were, for convenience of reference, given numbers prefixed by the letters TM.

II. THE FIRST STUDY — MOUNDS TM1 AND TM2

(a) *Description of Site and Mounds and Method of Recording Temperatures*

The work, begun in June 1933, was initiated with a search for two mounds similar in size, aspect, and isolation. Certain difficulties were encountered, which restricted the choice of mounds. Primarily it was necessary that the selected mounds should be within a short distance of each other so that readings could be made on both mounds with a minimum of delay between the readings in the respective mounds.

Two mounds which will be referred to as TM1 and TM2 were selected for the study. These two mounds were not a perfect pair, but were used for the study as a more suitable pair was not available at the time. The west wall of

mound TM2 contained a depression, a few inches in diameter, which suggested that the mound had been damaged in some way. Portion of another living mound of *E. exitiosus*, from the immediate neighbourhood, was placed in the cavity, in the hope that it would be incorporated in the mound and the damage repaired.*

It was decided that temperatures should be taken at similar points in each mound, and in the soil in the vicinity of each mound. Should the records show that the internal temperatures of these two mounds were similar, then it was proposed to kill the termites of one of the mounds and continue the temperature records of both mounds for a period to observe any change in temperature which might occur.

TABLE 1
PROBABLE INTERNAL DIMENSIONS OF MOUNDS TM1 AND TM2

Mound	Cross Diameters	Height	Thickness of the Regions of the Wall		Dimensions of Nursery in Vertical Section
			Outer Wall	Inner Wall	
1	3 ft. 0 in. x 3 ft. 2 in.	1 ft. 2 in.	4 in.	8 in.	8 in. x 10 in.
2	3 ft. 0 in. x 3 ft. 2 in.	1 ft. 4 in.	3 in.	4 in.	10 in. x 12 in.
3	3 ft. 0 in. x 3 ft. 0 in.	1 ft. 2 in.	3 in.	4 in.	11 in. x 12 in.
Average			3½ in.	5½ in.	10 in. x 11 in.

The two selected mounds were on the northerly aspect of a knoll of Black Mountain, Canberra, A.C.T. They were 45 yards apart and within 200 yards of the crest of the ridge, at an altitude of 2,100 feet. Both were situated on similar slopes and contours, but TM1, which was nearer the top of the ridge, was more likely to be influenced by the effects of cooling southerly winds.

Shrub growth around the two mounds was very similar, but saplings of scribbly gum (*Eucalyptus micrantha*) and stringy bark (*E. macrorrhyncha*) grew more densely around TM1. To ensure more equal insolation of the two mounds, about thirty saplings were removed from the northern sector of the territory of TM1, resulting in practically identical insolation of each of the two mounds throughout the greater portion of the day. Only early in the morning and late in the afternoon, when the sun's intensity was weak, was there any possibility of shadow from neighbouring trees being cast over the mounds.

*Andrews (1930) states that individuals from different colonies of *Termopsis angusticollis* can be mixed without any detrimental effect. Further, observations we have made suggest that in Nature, under certain conditions, colonies of *E. exitiosus* become united. It was felt, therefore, that the inclusion of a certain number of termites in the piece of mound transferred to TM2 would not be detrimental and the termites would tend to compensate for any loss of population that may have been associated with the damage to the mound. Subsequent examination of TM2 provided an explanation of this damage to the wall (see p. 477).

The similarity in size of the two mounds chosen is indicated by the following measurements. TM1 was 1 ft. 3 in. high and the cross diameters at ground level were 3 ft. 0 in. by 2 ft. 8 in. TM2 was 1 ft. 2½ in. high and the cross diameters at ground level were 3 ft. 1 in. and 3 ft. 0 in. From seventeen other mounds of this species of termite, three with external dimensions closest to TM1 and TM2 were selected, and the probable internal dimensions of the two mounds TM1 and TM2 were taken as the average of the internal dimensions of these three (see Table 1).

The regions of the mound are illustrated in Figure 1 and described in greater detail elsewhere (Holdaway 1933). The "nursery" occupied the middle of the mound and its centre is usually about ground level. It contains numerous thin-walled galleries capable of accommodating numbers of termites, and in it the termites congregate in large numbers in cold weather.

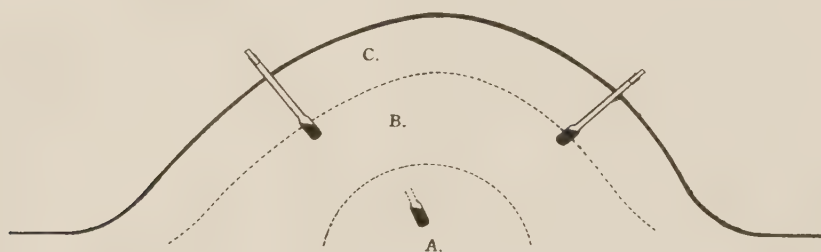


Fig. 1.—Diagrammatic section of mound TM1, showing temperature tubes in inner wall, north and south, and in the nursery.

A, nursery region; B, inner wall; and C, outer wall.

Glass tubes with an enlarged end containing mercury and into which thermometers could be placed were used for obtaining the temperature at the respective points in the mounds and soil. Tubes of five different lengths, according to the different requirements, were used, viz. 1 ft. 3 in., 11 in., 8 in., 6 in., and 4 in.; all tubes possessed a bulb 1 in. long. The internal diameter of the tubing was ⅜ in. and of the bulb ⅝ in. Clean mercury was placed in each bulb to a depth of 1 in., and the point of reading was taken as just above the centre of the mercury, approximately ⅝ in. from the bottom of the tube.

For each mound, readings were taken at five points in the mound and at three points in the surrounding soil. Those in the mound comprised one in the nursery and four in the wall. In the walls the tubes were installed to a depth of 4⅝ in. to give readings at approximately 4 in., which, according to the calculated internal dimensions of the mounds, is probably where the outer wall merges into the inner wall. The wall tubes were placed equidistant from the summit approximately half way from the summit to ground level and at the cardinal points. The nursery tubes were placed to give readings at a depth of 1 ft. near the centre of the nursery, and were inserted at a point equidistant from the north and east wall tubes, but somewhat nearer the summit (see Plate 1 which shows the position of the tubes; the nursery tube projects more than the other tubes).

Soil temperatures were taken at depths of 1 in., 5 in., and 8 in. respectively, near each mound. The tubes were installed in that order from the mound 4 in. apart on the south-east side and on the same contour, the nearest being 3 ft. 6 in. from the centre of the mound. The reasons governing the adoption of this distance from the mound and these depths are as follows. In field tests, timber samples are placed around the mound in a circle of radius 3-4 ft. Attack takes place mainly at a depth of 1 in. to 8 in. below ground level, with most samples experiencing initial attack at a depth of 5 in.

The open end of each temperature tube was closed with a rubber stopper. In the installation of all tubes, boring was done with a $\frac{3}{4}$ in. auger, the tube placed in the hole to the required depth, and wherever necessary, supported in position by soil or chips of wood. The tubes were installed between June 20 and June 30, 1933. By July 3, when the first readings were taken, all but two of the tubes had been built firmly into position by the termites, and the remaining two were consolidated in this manner by July 12 to July 14.

A standard meteorological screen was erected on the south side of TM1 at a distance of 5 ft. 6 in. from the mound. A thermograph giving continuous weekly records was maintained in this cabinet throughout the study.

In the original plan it was proposed to take readings four times daily, at 9 a.m., 11.30 a.m., 2 p.m., and 4.30 p.m. respectively, for five consecutive days (Monday to Friday) every four weeks. This plan was adopted for the first few months, but it was then found necessary to make additional readings which will be mentioned later.

Readings taken were: temperature in the mounds, soil, and air; wind velocity and direction; nebulousity; insolation and rainfall.

Thermometers checked against a standard thermometer were used in taking mound and soil temperatures. Air temperatures were obtained from the thermograph, which was checked at frequent intervals. Wind velocity was read from the Beaufort scale, direction being obtained from a vane erected near the meteorological screen. Nebulousity was estimated on a scale 0-10, representing solely the proportion of clear sky to cloud, taking no account of the density of the clouds present. Daily insolation records were obtained with a Campbell Stokes Sunshine Recorder at the C.S.I.R. laboratories about $\frac{3}{4}$ mile from the site of the study. Rainfall records were obtained also from the laboratory.

(b) Temperature Records from TM1 and TM2

The temperature records obtained during the first week, i.e. July 3 to July 7 inclusive,* showed that a constant temperature was not maintained in the mounds, that the nursery was warmer than the air, the soil, and the mound wall (even those portions of the wall receiving the greatest amount of sunshine).

Certain of the temperature readings obtained during this week, and also during the second period when observations were made (July 24 to July 28 inclusive) are shown graphically in Figure 2. The readings from the mound wall and the soil are omitted. The temperature changes and trends in the different

* Records for July 5 were incomplete because of rain.

parts of the mounds and at different depths in the soil were indicated more fully and satisfactorily by the continuous records which were obtained later. These changes and trends will be discussed with the continuous records below. It is sufficient to mention here that such differences in detail as are manifest in the two sets of records (those taken in July and the continuous records of August 8 to 10) are readily explained by differences in weather conditions during the two periods.

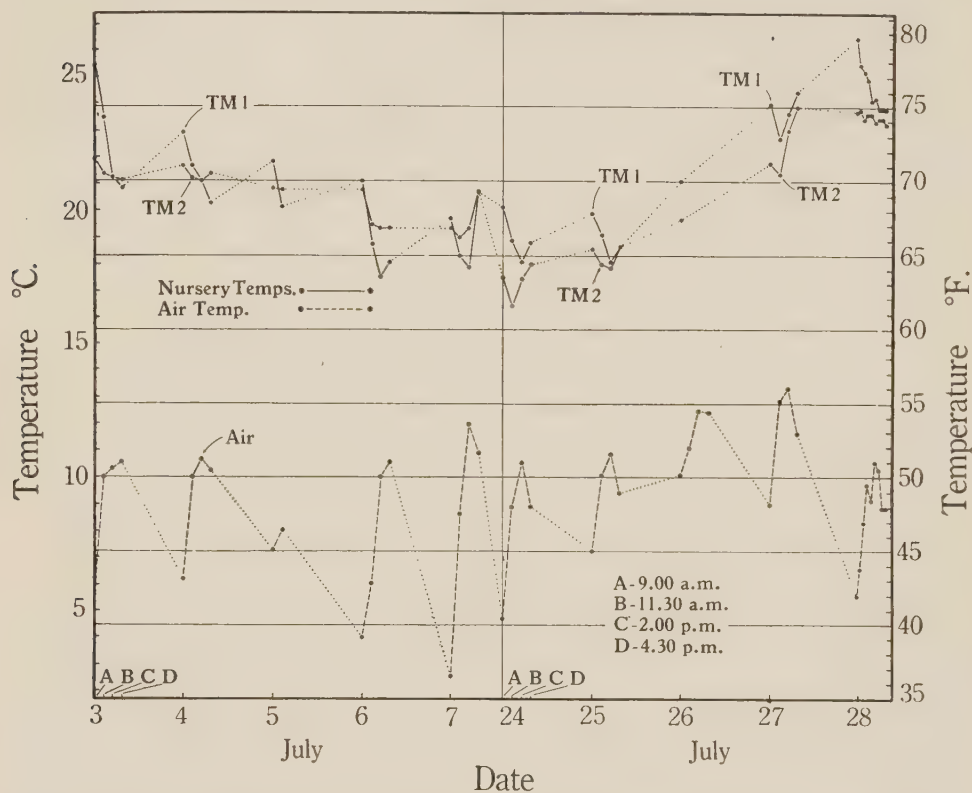


Fig. 2.—The nursery temperature of mounds TM1 and TM2 during the first two weeks in which observations were made, together with the air temperature for the same period. Readings taken on successive days are joined by dotted lines.

Although the general order of magnitude of the nursery temperature was similar in both mounds, it will be seen from Figure 2 that the temperature range during the day was greater in TM1 than in TM2. At the time no explanation of this fact offered itself. Later it was found that the constitution of the populations of the two mounds differed considerably (see p. 477), and the probable explanation of the differences between the temperature ranges was obtained.

In order to acquire additional and more complete information on the temperature of the mound and its habitat, it was decided to obtain continuous hourly readings over a period of at least two days. Actually they were obtained between 8 a.m. on August 8 and 4.30 p.m. on August 10, a period of 56 hours.

No rain had fallen since August 2, when 8 points fell. From August 6 up to and including the period of continuous records there was little cloud during the day, the sun shining most of the time.* During the daytime there was a little movement of the air: at times the air movement rose to a slight breeze (4-7 m.p.h.), increasing at times on August 6 and 8 to gusts of gentle breeze (8-12 m.p.h.). The nights were calm and clear. Frosts occurred on the mornings of August 6 and 7. There was a heavy frost on the morning of August 8, but it did not occur on the ground immediately surrounding the mounds. No frost occurred on August 9, but there was a light frost on the morning of August 10.

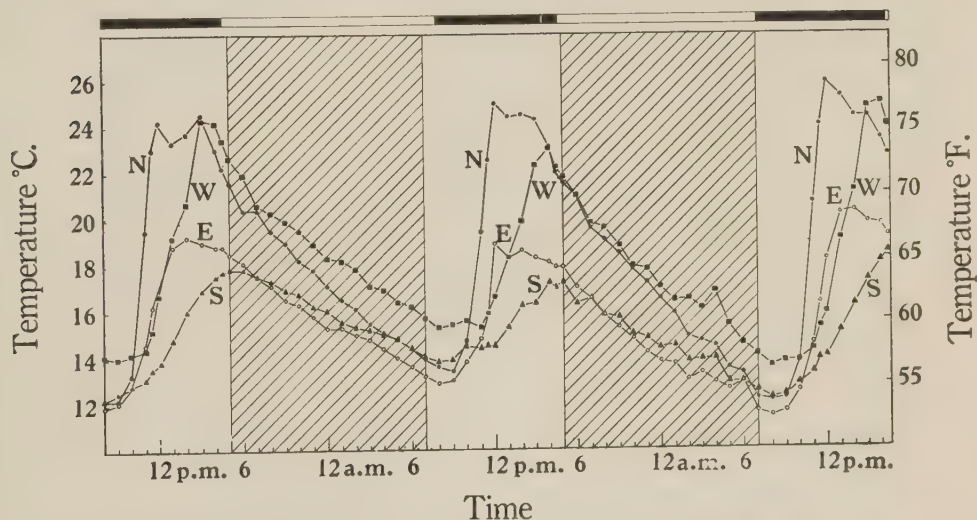


Fig. 3.—The temperature at the cardinal points in the inner wall of TM1 over a period of 56 hours, August 8-10, 1933. Hours of sunshine are given in the block diagram at the top of the figure. Darkness is shown by cross hatching.

The records of the temperature changes in TM1, the soil, and the air, obtained during the continuous observation period, are presented graphically in Figures 3 and 4. From these data two important points emerge:

- (1) The nursery, although not maintaining a constant temperature, was continuously much warmer than either the air or the soil, a fact which it would be impossible to explain except on the supposition that the high temperature was due in some way to the termites. Experimental confirmation of this supposition was obtained later.
- (2) The temperatures in the different parts of the mound and at different depths in the soil showed rhythmic changes which must have been governed by changes in the air temperature.

The correlation between the fluctuations of mound and soil temperatures and the air temperature, however, was markedly affected, both as regards magnitude and time by (a) insolation, and (b) distance from the surface.

* On August 6 and 7 there were 6.3 and 8.3 hours of sunshine; and on August 8, 9, and 10, 9.4, 8.3, and 9.6 hours respectively.

The effect of insolation is clearly manifest in the temperature records of the four aspects of the mound wall (see Fig. 3). From 9 a.m. onwards the temperature of the north wall, which received the greatest insolation, rose more rapidly, and to a higher level, than did the temperature of any other aspect of the wall. The maximum temperature recorded by each portion of the mound wall bore an obvious and direct relation to the amount of insolation which it received; moreover, the various aspects of the wall attained their maximum temperatures progressively later in the day as the sun's rays struck them more directly. The order of reaching a maximum temperature was, in general, east, north, west, south — the east between noon and 2 p.m., the north between noon and 3 p.m., the west between 3 p.m. and 4 p.m., and the south between 4 p.m. and 6 p.m. The temperatures recorded in the different parts of the mound wall (approximately 4 in. in) were intermediate between the temperatures in the nursery and in the soil.

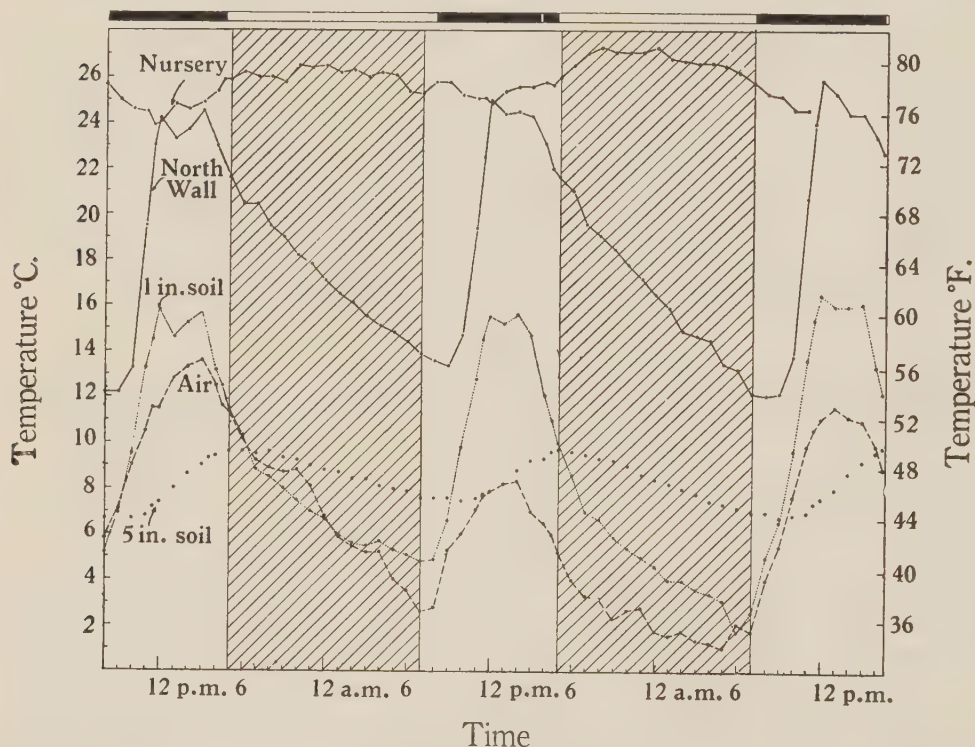


Fig. 4.—The trend of temperatures in the nursery and north wall of TM1 and in the neighbouring soil; the fact that the nursery was continuously much warmer than the soil and warmer than the highest temperature recorded in the warmest portion of the wall, viz. the northern portion (August 8-10, 1933) is illustrated.

During the night the mound wall cooled down rapidly, the north portion falling in temperature most rapidly and the south least rapidly. Towards the time of minimum wall temperatures, which usually occurred between 8 a.m. and 9 a.m., the west and south walls were warmer than the east and north.

The records of the temperature changes at different depths in the soil (see Fig. 4) show the effect both of insolation and of distance from the surface. Between the air and the soil at 1 in. the only significant difference was that the soil attained a higher temperature, undoubtedly as a result of the heating effect of the sun's rays. At a greater depth in the soil the temperature fluctuates less than at the surface, and in its changes lags noticeably behind the air temperature; thus, while the air temperature reached its maximum in the early or mid-afternoon, the maximum temperature of the soil at a depth of 5 in. did not occur until sunset, and at a depth of 8 in. (not shown in the figure) the maximum occurred about an hour later.

The temperature fluctuation in the nursery was very similar to that in the soil at a depth of 8 in., except that it was at a much higher level (the mean nursery temperature was 31.2°F. higher than that of the soil at 8 in., and 32°F. higher than the mean temperature of the soil at 5 in.).

The magnitude of the daily oscillation averaged 4.3°F. in the nursery and 4.7°F. at a depth of 8 in. in the soil, and the daily maxima and minima in the nursery occurred at about the same time as, or a few hours later than, those in the soil at 8 in.

Because the temperature changes in the nursery lag behind those in the environment even more than do those of the soil at a depth of 8 in., there is a striking difference between the temperature curves of the nursery and the mound wall. The nursery reached its maximum between 8 p.m. and midnight, when air and wall temperatures were falling fairly rapidly; and when the nursery was approaching its minimum, the east and north walls were approaching their maximum temperatures.

A point that deserves special mention in connection with the nursery is that the temperature at 9 a.m. was a fairly close approximation to the average for the day,* a fact which was utilized in a later experiment (see p. 479).

Neither the records obtained during the period of continuous observation, nor those obtained prior to this and shown graphically in Figure 2, give a satisfactory indication of the relationship that exists between the temperatures of the nursery and of the air. This relationship is brought out very clearly, however, in Figure 5, in which the mean temperatures of the nursery and of the air over a twelve months' period are shown. It will be seen that the annual air temperature cycle is closely reflected in the nursery temperature of TM1. (Records of TM2 can be disregarded at this stage. They were affected by an event which will be discussed in detail in the next subsection, and which rendered them of no value in a study of the temperature relations of a normal mound.)

* In standard meteorological practice, when it is only possible to obtain one record each day, 8.00 a.m. has been accepted internationally as giving the best approximation to the daily temperature average. Professor J. A. Prescott, former Chief of the Division of Soils, C.S.I.R., informed the writers that some years ago the Commonwealth Bureau of Meteorology decided that readings taken at 9.00 a.m. gave, in Australia, a more satisfactory approximation to the daily mean.

Although the correlation between nursery and air temperatures affects only the major trends, and requires long-term records for its demonstration, the data obtained over short periods did indicate that environmental conditions could have a demonstrable effect on the nursery temperature. Thus during the period when continuous records were being taken (August 8 to 10) — two days of almost continuous sunshine — the minimum nursery temperature occurred just before noon; but on July 3 and 4 (see Fig. 2) the temperature of the nursery fell continuously until 4.30 p.m. Examination of the sunshine and nebosity records reveals that these were both dull days. (On July 3 the sunshine in hours was nil and the nebosity 10; on July 4 the figures were 3.8 hours and 8 respectively.)

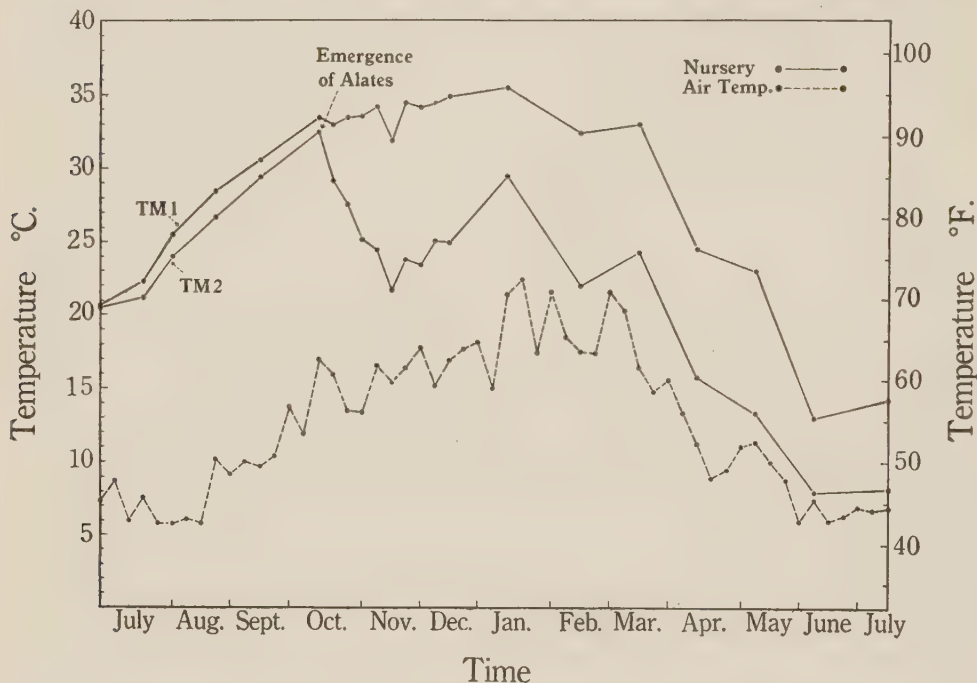


Fig. 5.—The seasonal trend of the mean nursery temperature of mounds TM1 and TM2; also the drop in temperature of TM2 associated with emergence of alates and the approach of the temperature of TM2 to that of the environment at the end of the period, when the mound was dead.

On July 6 and 7, when the sunshine increased and the nebosity decreased, there was a rise in nursery temperature between 2 p.m. and 4.30 p.m. Similarly, records obtained later in the year, under early summer conditions (at the end of November), showed that a small amount of sunshine associated with high nebosity resulted in the nursery temperature not reaching a minimum until late in the afternoon.

During mid- and late winter, the temperatures of the mound wall were consistently lower than that of the nursery, except that in August, for a brief period near midday, the north wall rose to the temperature of the nursery (see Fig. 4). Under full summer conditions, as will be seen from Figure 6, this was

no longer the case; and the temperature of the warmer parts of the wall rose considerably higher than the nursery temperature during the latter part of each day. This fact lends support to the supposition that the high temperature of the nursery is due, not only to heat absorbed from the walls as they are warmed by the sun, but to the presence of living termites within it. Were this not so, the

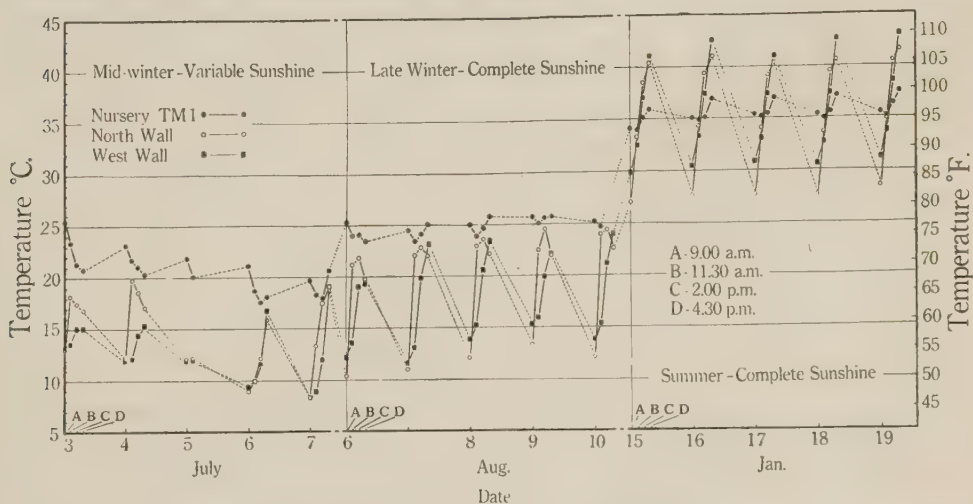


Fig. 6.—The relation between wall temperatures (N. and W.) and nursery temperature under various conditions. Readings taken on successive days for each of the three positions are linked by broken lines. Dotted lines link the observations made at the different periods. (i) Various amounts of sunshine in mid-winter. (ii) Complete sunshine in late winter. (iii) Complete sunshine in summer.

nursery temperature would bear a constant general relationship to the wall temperature, and under summer conditions would rise to a higher level than it actually does.

(c) *The Emergence of Alates from TM2, and the Condition of TM1 and TM2 at the End of One Year*

During the first few months in which records were taken, the trends of the mean nursery temperatures of TM1 and TM2 were similar: the main difference between the two appeared to be that the temperature in TM2 was consistently lower by approximately the same amount. It therefore seemed that the two mounds were sufficiently similar to be satisfactory for the purpose for which they were selected, namely, the study of the temperature of a "living" mound and a "dead" mound which, during life, had a temperature comparable with that of the mound in which the termites were allowed to remain alive. Further, it appeared that the similarity in temperature of the two mounds justified the selection of TM2, although slightly damaged, for this particular temperature study. Events which followed, however, showed that TM2 could no longer be considered satisfactory. Nevertheless, these events contributed to the understanding of the temperature relations of mounds.

On the night between October 23 and October 24, after the temperatures of the two nurseries had followed similar trends at comparable levels for nearly four months, alates emerged from TM2. On the morning of October 24, large numbers of wings were found around the base of the mound. Others had been built into the walls when the emergence holes had been closed up by the workers. It had been noted that on October 23 the temperature of the mound had risen appreciably: it actually rose to 92.9°F., the highest temperature recorded to date in this mound, and equalling that of TM1, which had previously been consistently higher than TM2.

Immediately after the emergence of alates from TM2 the temperature of the nursery fell rapidly, and continued to fall for some days. At 9 a.m. on October 26, approximately 36 hours after emergence, the nursery temperature of TM2 was more than 15°F. lower than that of TM1; whereas on October 23 the difference had been only 2.8°F. After the emergence of alates the weather became cooler; but over the four-day period when the temperature of TM2 dropped 18.3°F., the temperature of TM1 dropped only 4.9°F.

After the fall in temperature associated with the emergence of alates from TM2, and the cool change which occurred a few days later, the nursery temperature began to rise, but throughout the remainder of the year, it never again reached a temperature comparable to that of TM1 (see Fig. 5). From the middle of summer it followed a similar trend to that of the nursery temperature of TM1, but at a much lower level.

The marked fall in the nursery temperature of TM2 following the emergence of alates suggested that the temperature of a mound is affected by the presence within it of nymphs* or alates. Elsewhere (Holdaway, Gay, and Greaves 1935) it has been shown that there may be as many as 50,000 alates present in the mound at the same time. The mound temperature would almost certainly be markedly affected by the presence within it of thousands of large-bodied alates, whose departure would be reflected in a reduction in mound temperature. The smaller range in temperature of TM2, already noted, was probably due to the fact that the large-bodied individuals were not capable of rapid movement within the mound. The reason for the temperature of TM2 being consistently lower than that of TM1, although the former contained a large number of alates, was made clear when it was discovered (as will be explained below) that the strength of the colony in TM2 had been depleted before the study began. The great difference in the population strengths of the two mounds after the emergence of alates accounted for the much lower level in the temperature of TM2 from November onward.

From the accumulated data on the colonizing flight of *E. exitiosus*, it appears that the emergence of alates occurs between 8 p.m. and midnight, commonly at about 10 p.m. After the emergence of alates from TM2, on several occasions when environmental temperatures and mound temperatures were rising, observations on TM1 were continued throughout the night or until midnight in the

* The term "nymph" is used here in the restricted sense as applying only to those developmental stages of the winged reproductive caste in which wing pads are evident.

hope of observing actual emergence, and of securing data on the temperature within the mound prior to, during, and after the emergence of alates. Emergence was never observed. By the end of the year it was decided that the mound probably had had no alates to yield. Examination of the temperature graph of TM1 (Fig. 5) shows that a drop in the nursery temperature occurred over the week-end November 17-20. This might have been interpreted as indicating an emergence of alates from TM1 during this period; but a similar drop in the nursery temperature occurred in TM2, which had already yielded alates. As 120 points of rain fell during this week-end, the drop in temperature noted in both mounds was more probably due to the cooling effect of rain, an effect noted several times during observations.

At the expiration of one year and three months, both mounds were examined in detail, TM2 on August 2, 1934, and TM1 on August 6, 1934. Some time before this, the external appearance of TM2 suggested that its colony was dead, or at any rate greatly reduced in numbers. Further, the piece of mound material placed in the damaged portion of the mound had not been completely incorporated. Only four living termites were found in TM2, all of them being workers. In the nursery were discovered the remains of twelve egg-like bodies and the shrivelled remains of six small lizards. These were later identified as the young of the Monitor lizard *Varanus varius* Shaw — the so-called "goanna."

J. R. Kinghorn, of the Australian Museum, Sydney (personal communication), stated that oviposition in termite mounds has been recorded before. Cowles (1930) has given an account of the oviposition of the "Qamu" or Nile Monitor, *Varanus niloticus* Linn., in mounds of *Nasutitermes trinerviformis* Holm. in Natal. From his observations on the Nile Monitor, it would appear that the damage to TM2 noted at the beginning of the observations was due to damage made by the female goanna when the eggs were deposited within the mound. As far as is known *Eutermes exitiosus* does not produce supplementary sexuals. From the behaviour of the colony and its ultimate death, it would appear that the queen termite had been killed either at the time the eggs of the goanna were deposited in the mound or when the young lizards emerged from them. Thus, when nymphs present in the mound matured to alates, the colony, reduced in numbers during the deposition of the goanna eggs, and devoid of any means of increase, gradually died out.

The examination of TM1 showed that some of the colony had succumbed as a result of the breaking of one of the temperature tubes on December 13, 1933, and the resultant entry of mercury into the mound (see Fig. 5 which shows that the temperature of this mound approached the air temperature more closely after the date of the accident to the temperature tube than before). An estimation of the population present in the mound was made by the method previously described (Holdaway, Gay, and Greaves 1935). The population estimated to be present in this mound was approximately 527,500. This figure is considerably lower than that estimated for mounds of similar size or very slightly larger than TM1.

III. THE SECOND STUDY, A "LIVING" MOUND AND A "DEAD" MOUND UNDER WINTER CONDITIONS—MOUNDS TM3 AND TM4

The unexpected behaviour of the colony associated with TM2, and its subsequent death, prevented the employment of the original pair of mounds for the completion of the programme which had been outlined. A further pair was therefore selected in May 1934. These mounds, TM3 and TM4, were both situated on a slight NNE. slope in open forest country, TM4 being higher up the slope on the same aspect line and about 5 ft. above TM3. The measurements of the two mounds were as follows: Mound TM3, 1 ft. 6 in. high and 3 ft. 0 in. x 3 ft. 3 in. horizontal dimensions at ground level, mound TM4, 1 ft. 5 in. high and 3 ft. 3 in. x 3 ft. 10 in. horizontal dimensions at ground level.

Observations made on TM2 suggested that the most satisfactory results would be secured if both the mounds selected for the study contained similar castes. The two mounds were therefore examined to determine which castes were present. As insolation is from the north, the examination for castes was made on the southern section of the mounds, so that any slight disturbance to the mound would affect the mound temperature to a minimum degree. Moreover, in the early portion of the day before the effect of the sun's rays has become marked, the southern wall is relatively warmer than other portions of a mound and would be expected to contain an accumulation of termites.

On the morning of June 18, a small portion of the outer wall on the southern aspect near ground level was carefully removed from each mound. Small pieces of the inner wall were then removed from below ground level towards the centre of the mound, until the desired information on the castes was secured. In both mounds, workers and nymphs were observed, although nymphs were not found as readily in TM3 as in TM4. In the search for nymphs, TM3 was necessarily damaged somewhat more than TM4. It will be seen later that the temperature of TM4 was higher than that of TM3. This is possibly due to a larger number of nymphs present in TM4, which would account for their being found more readily in TM4 than in TM3.

Temperature tubes were inserted in the mounds and soil as for TM1 and TM2 except that the nursery tube was inserted through the top of the mound. A two-point mercury-actuated distance-recording thermometer was installed at TM3 (the mound to be killed), one bulb in the centre of the nursery against the nursery temperature tube, and one at a depth of 5 in. in the soil against the 5 in. soil temperature tube. The meteorological cabinet used in the study of TM1 and TM2 was erected to the south of TM3 and contained a thermograph for recording the air temperature and the recorder for the distance thermometers. The flexible lead tube of the thermometer bulb, inserted in the nursery, entered the mound about ground level on the south-west section of the mound. From the mound it passed back for about 4 ft., through a protective earth-work about 5 in. high, to the cabinet and thence up through the bottom of the cabinet to the recording instrument. The bulb inserted at 5 in. in the soil was to the south-east of the mound, the lead tubing running at a depth of 5 in. through the soil to the cabinet.

The temperature tubes and bulbs of the distance recording thermometers were placed in position on June 18 immediately after the examination for castes.

On June 21 several saplings were felled in the vicinity of both mounds to ensure comparable insolation in the two habitats. By June 27 the nursery and the south wall tubes of TM3, and the nursery and the east, north, and west wall tubes of TM4, had been built into the respective positions. The other tubes were built in during the next few days.

It was found that the records from the distance recorder indicated the trends of temperature but the temperature tubes permitted rapid changes of temperature to be observed better than did the distance recorder.

It was felt that in destroying the colony associated with TM3 it would be unwise to employ the usual method of introducing the poison through the upper portion of the mound (Holdaway and Hill 1936). The application hole would not be repaired completely before the death of the colony and would thus permit temperature changes other than those which would occur in a mound with a completely undamaged surface. It was therefore decided to introduce the poison into the nursery from below ground level and thus avoid damage to the outer wall. This was done in two mounds similar in size and situation to TM3. White arsenic was introduced into the nurseries of these mounds by way of a trench dug adjacent to them. No damage was done to the outer walls, and it was found that most of the termites were dead one month after treatment. The method was, therefore, deemed satisfactory for the purposes of this study.

During the weeks following the installation of temperature tubes and recording thermometer bulbs, the trend of temperatures in one of the mounds, TM3, and in the soil was followed on the distance recorder charts. At intervals, complete records of the temperatures of both mounds and of the soil were made. On July 16, four weeks after the installation of the temperature tubes, regular records were taken four times daily. The two mounds were found to have comparable temperatures although that of TM4 was from 4° to 8.5°F., or an average of 6.4°F., higher than that of TM3.

At 10.45 a.m. on July 19, 1 oz. of white arsenic was applied to TM3 from below the nursery by the method already described. The daily records were continued until the end of the week. At intervals during the succeeding weeks records were made at 9 a.m. Beginning on September 3, just over six weeks from the time of the treatment of TM3, by which time it was judged the mound would be dead, the regular records were made for two days. On September 7 the mound was opened and the colony found to be extinct. Dead termites were massed in the bottom of the mound 1 ft. 10 in. from the apex, in the manner characteristic of colonies destroyed by arsenical powders. The poison had been delivered to the centre of the nursery 1 ft. from the apex of the mound; arsenic was still evident. Mound TM4 was examined the same day and an estimate made of the population present in the mound. There were approximately 797,000 termites, of which 2.25 per cent. or approximately 18,000 were nymphs.

During the few days prior to the application of arsenic, the temperature of the two mounds at 9 a.m. differed by approximately 9.2°F. After six weeks,

when TM3 was dead, the temperature differed by from 22.7° to 24.7°F. , indicating an average difference due to the absence of termites of 14.5°F.

The fall in temperature after the death of the mound is illustrated further by comparing the temperature of TM3 with that of the soil in the vicinity of it (see Fig. 7, which gives the mean daily temperature of the mound in comparison with that of the soil at a depth of 5 in.). Prior to the application of arsenic the mound was considerably warmer than the soil. After the application of arsenic, the temperature of the mound fell rapidly for three days, when it settled down to follow a trend very similar to, but on the average 2°F. lower than, the tempera-

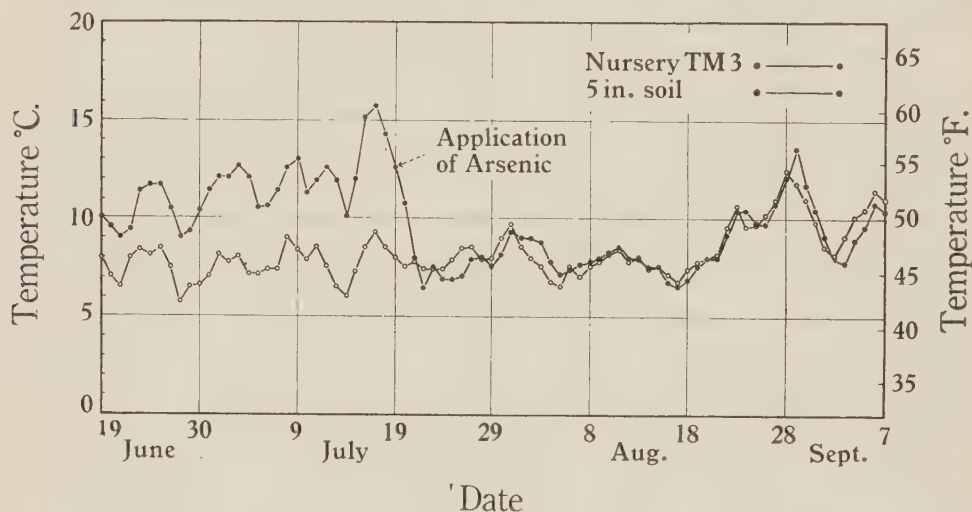


Fig. 7.—The temperature of the nursery of TM3 and that of the soil at 5 in. depth prior to, and after, death of TM3 are compared. Following death of the colony, the temperature of the nursery approximates to that of the soil. Daily average temperatures.

ture of the soil at a depth of 5 in. At intervals between the third day after application and the death of the complete colony, the mean mound temperature rose above the mean soil temperature. It would appear that most of the termites present in the mound died within six days of the application of the arsenic, and that the periodic rises in mound temperature marked the re-entry into the mound of termites which were present in galleries away from the mound at the time the arsenic was applied.

The second study of mound temperatures thus demonstrates that, under winter conditions, the temperature of the mound is affected by the presence of living termites in it, and that populations of approximately 800,000 termites, in mounds with cross diameters at ground level of approximately 3 ft., are capable of raising the temperature from 13.5° to 15.5°F. above that of an unoccupied mound.

IV. THE THIRD STUDY, A "LIVING" MOUND AND A "DEAD" MOUND UNDER SUMMER CONDITIONS — MOUNDS TM6 AND TM7

A further study similar to that made under winter conditions was made under summer conditions. The mounds for this study, TM6 and TM7, were

selected in August 1935. Both were situated in open forest country, and were similar in size and situated on slopes of similar aspect. Records of nursery temperatures, made on August 23, before the installation of temperature tubes, showed the two mounds to have similar temperatures, TM6 at 3 p.m. being 83.3°F., and TM7 at 3.30 p.m. being 80.8°F.

A second distance recorder, one with a single bulb, was available for this study. The two-point recorder was installed at TM6, as at TM3, and the single-point recorder in the nursery of TM7. Temperature tubes were installed only in the respective nurseries, since it was apparent by this time that the most important records from the mound were those from the nursery. The temperature

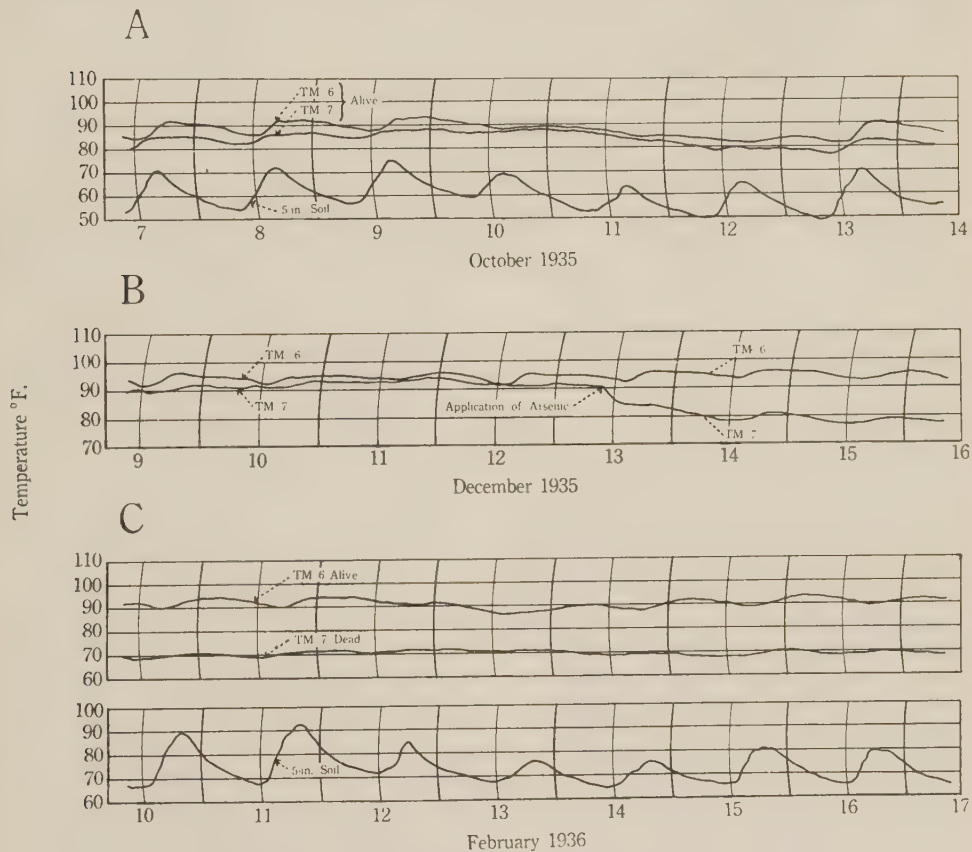


Fig. 8.—Continuous records of nursery temperatures of TM6 and TM7.

A. When both colonies were alive.

B. During the week when arsenic was applied.

C. When TM7 was dead.

The records have been transposed to a single chart and synchronized, and in A and C, records of the soil temperature at a depth of 5 in. have also been included.

tubes installed in the nurseries were used very little, the information being obtained mainly from the weekly continuous temperature records of the nurseries, air, and soil at a depth of 5 in. The two-point distance recorder, with one bulb

in the nursery of TM6 and the other in the soil at a depth of 5 in., was housed with the thermograph recording air temperature in the meteorological screen at TM6. The single-point distance recorder, with bulb embedded in the nursery of TM7, was housed in a temporary protective cabinet. Saplings were removed from the environs of both mounds to equalize insolation.

Records of the temperatures in the two mounds during the early summer indicated that the temperatures of both mounds were following similar trends, but TM7 was always a few degrees below TM6. The range in temperature in each mound over a given period of time was similar. Figure 8A shows the continuous temperature records of the two mounds, when both were alive, during a typical week in early summer. The range in temperature over this week was the same in both mounds, namely, 11°F.

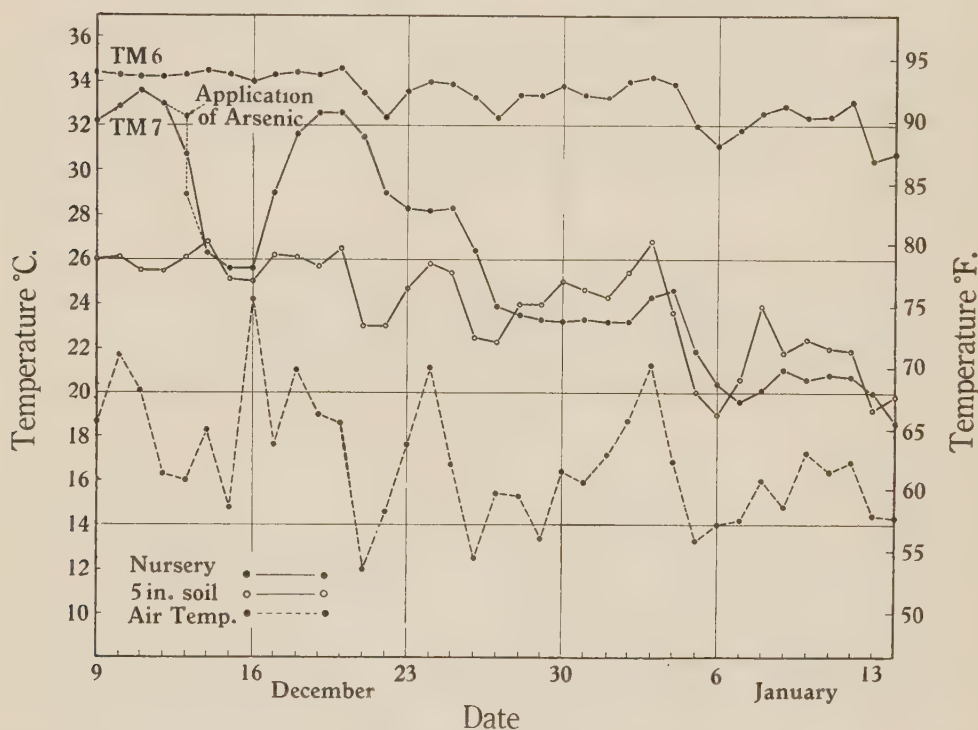


Fig. 9.—The nursery temperatures of mound TM6 and mound TM7 prior to, and after, death of TM7 are compared. Note the fall in temperature of TM7 following the application of arsenic, and the subsequent rise in temperature. The temperatures are daily averages. The three readings shown on the day of treatment are as follows. The highest reading is the mean temperature for that portion of the day up to the time of treatment, the mid-reading is the average for the day, whilst the lowest reading is the mean for that portion of the day following the time of treatment.

On December 13, 1 oz. of white arsenic was applied to the nursery of TM7 at 9.50 a.m. by the method already described and used for TM3. On January 13, 1936, one month after treatment, TM7 showed signs of weathering. The colony was apparently extinct by this time. Its average temperature on January 14 was

65.5°F. and that of TM6, which was still alive, was 87.2°F. Figure 8B gives the continuous records of the nursery temperatures during the week that arsenic was applied to TM7, and Figure 8C, the records for a week when TM7 was dead.

Figure 9 shows the temperature changes in the two mounds just prior to the treatment of TM7, and during the succeeding weeks. It will be seen that immediately following the application of poison to TM7 the temperature fell rapidly. After three days, however, the temperature rose sharply again, so that, six days after treatment, the mound was as warm as it had been at the time of treatment. This rise in temperature, however, proved temporary only. While just before treatment the difference in the nursery temperatures of the two mounds averaged 2.6°F., one month after treatment the difference in the temperature of the living mound, TM6, and the dead mound, TM7, was 21.2°F. (average taken from the three consecutive days, January 12 to 14 inclusive). Thus the difference in temperature due to the presence of living termites was approximately 18.6°F.

Although the behaviour of the two mounds, TM3 and TM7, treated under winter and summer conditions respectively, was on the whole very similar, there were nevertheless certain points of difference. In both mounds the temperature fell for some days immediately after treatment; but the fall was greater and more rapid in TM7 which was treated under summer conditions. (The total drop in temperature in the three days after treatment was 13.4°F. in TM7, and 9.5°F. in TM3.) In both mounds the temperature rose on the fourth day after treatment; but in TM3 the rise was slight — less than 2°F.— and did not continue, while in TM7 the rise continued for three days, and was almost as great (namely 12.7°F.) as the drop in temperature following treatment. After this, in both mounds there were recurring rises of temperature above that of the soil at a depth of 5 in.

The increase in temperature due to the presence of living termites in the mound was not markedly different under winter and under summer conditions, being 14.5°F. in the winter and 18.6°F. in the summer.

Observations made in connection with other investigations on termites (Holdaway, Gay, and Greaves 1935) have shown that during the winter months there is a much greater concentration of termites in the mound than during the summer. Since a lower population in a mound in the summer is capable of maintaining the mound temperature as much above that of an unoccupied mound as is the much higher winter population, it must be concluded that the greater rise in temperature per unit of the population under summer conditions is due to the higher metabolism associated with summer temperatures. The low summer population in the mound would also explain the greater and more rapid temperature fall following treatment in TM7 than in TM3, for the fewer termites would presumably be killed more quickly. Possibly, however, the toxic effect of arsenic might be more rapid at summer temperatures with their associated high rate of metabolism.

The recurring rises in temperature following treatment are apparently due to the return of termites which were absent from the mound when the poison was introduced. The more marked rise in temperature in TM7, between the

fourth and the seventh day after treatment, presumably was due to the fact that under summer conditions a relatively large percentage of the colony would be in galleries away from the mound and therefore larger numbers would return (as presumably they would tend to do) when an interruption of normal activity indicated that all was not well with the nest colony.

The temperature data for the week, October 7 to 14, 1935, presented graphically in Figure 8A, show that the range in temperature within the two mounds, TM6 and TM7, during this week was the same, namely, 11°F. The data for the week, February 10 to 17, when TM7 was dead, are given in Figure 8C, and show that the range in temperature within the living mound, TM6, was 8°F. (86°-94°F.) whilst the range in the dead mound, TM7, was only 3°F. (68°-71°F.). In addition, the latter figure shows the oscillations in temperature of the soil at a depth of 5 in. The range of temperature at this depth was 28.75°F. (64.25°-92°F.).

Thus, while the temperature is dependent on the living termites, there is less variation within an unoccupied mound than in an occupied mound. It is of interest to consider the possible reasons for this. Both moist soil and wood conduct heat better than does dry soil or wood. Since the material of which the mound is composed is a mixture of earthy material and partially digested wood, it might be expected to behave in much the same way as soil or wood as regards the relation of moisture content to the passage of heat. Records of the moisture content of mound material over a number of years, together with the evidence secured by Fyfe and Gay (1938), show that the moisture content of the material

TABLE 2
MOISTURE CONTENT OF THE MOUND MATERIAL OF TM6, OCCUPIED, AND TM7, UNOCCUPIED

Region	Mound	Moisture Content (Mean of 3 Readings) (%)
Inner Wall	TM6 (occupied)	30.2
	TM7 (unoccupied)	34.0
Nursery	TM6 (occupied)	36.3
	TM7 (unoccupied)	44.2

of the inner wall of an occupied mound is comparatively high, and that the humidity of the mound atmosphere is also high, 95-98 per cent. Thus, since the occupied mound shows a greater range of temperature than the unoccupied mound, and has a comparatively high moisture content, one might expect the lower temperature range of the unoccupied mound to be due to a lower moisture content, as a result of the absence of moisture of metabolism. Moisture content determinations of the mound material of both mounds showed, however, that the unoccupied mound had a *higher* moisture content than the occupied mound (see Table 2), apparently due to the fact that the mass of dead termites in the mound gave off even more moisture in the early period following death of the colony than did the living termites. (This suggestion is in accordance with the writers' observations in laboratory colonies.) Thus, by virtue of its higher moisture

content the unoccupied mound might be expected to show a *greater* fluctuation in temperature than the occupied mound. Since the unoccupied mound shows less fluctuation in temperature than the occupied mound, some factor other than moisture content must be responsible for the difference between the two mounds.

It is known that the termites concentrate in different portions of the mound at different times of the day. This is particularly noticeable in winter when the termites concentrate in those portions of the mound receiving the greatest insolation. It has already been seen that the termites, by means of their metabolism, raise the temperature of the mound they occupy. It is conceivable, therefore, that in moving from one portion of a mound to another they raise the temperature of the portion of the mound into which they move. Thus the greater fluctuation in temperature within the occupied mound would probably be due to the movements of the termites within the mound. (This idea has already been advanced in explanation of the difference in temperature fluctuations of mounds TM1 and TM2 noted during the first week of observations.)

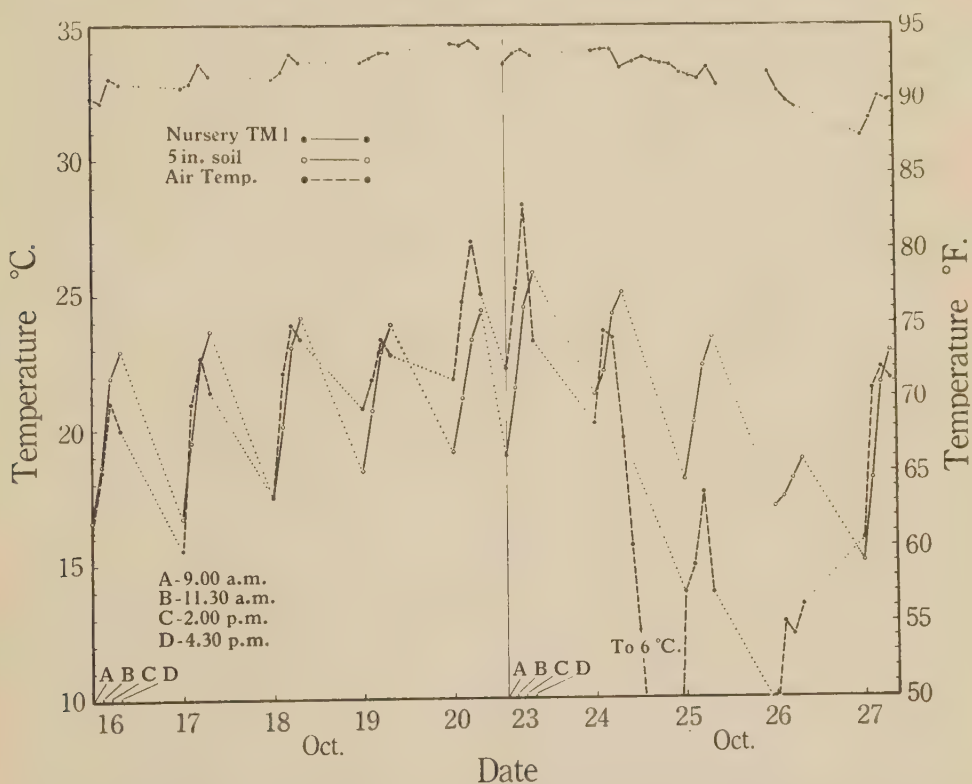


Fig. 10.—The comparatively small drop in nursery temperature associated with a marked drop in environmental temperature at a season of the year when a large number of termites is present in the mound is shown. Readings taken on successive days are linked by dotted lines.

While a reason has been suggested for the occupied mound showing a greater fluctuation in temperature than the unoccupied mound, it has still not

been shown why an unoccupied mound has, over a limited time, so uniform a temperature. The mound is raised above the surface of the soil and presents a considerable amount of exposed surface which might be expected to result in marked fluctuations in temperature. Nevertheless, soil at a depth of 5 in. shows, over a period of one week, a temperature range of 28.75°F. while the

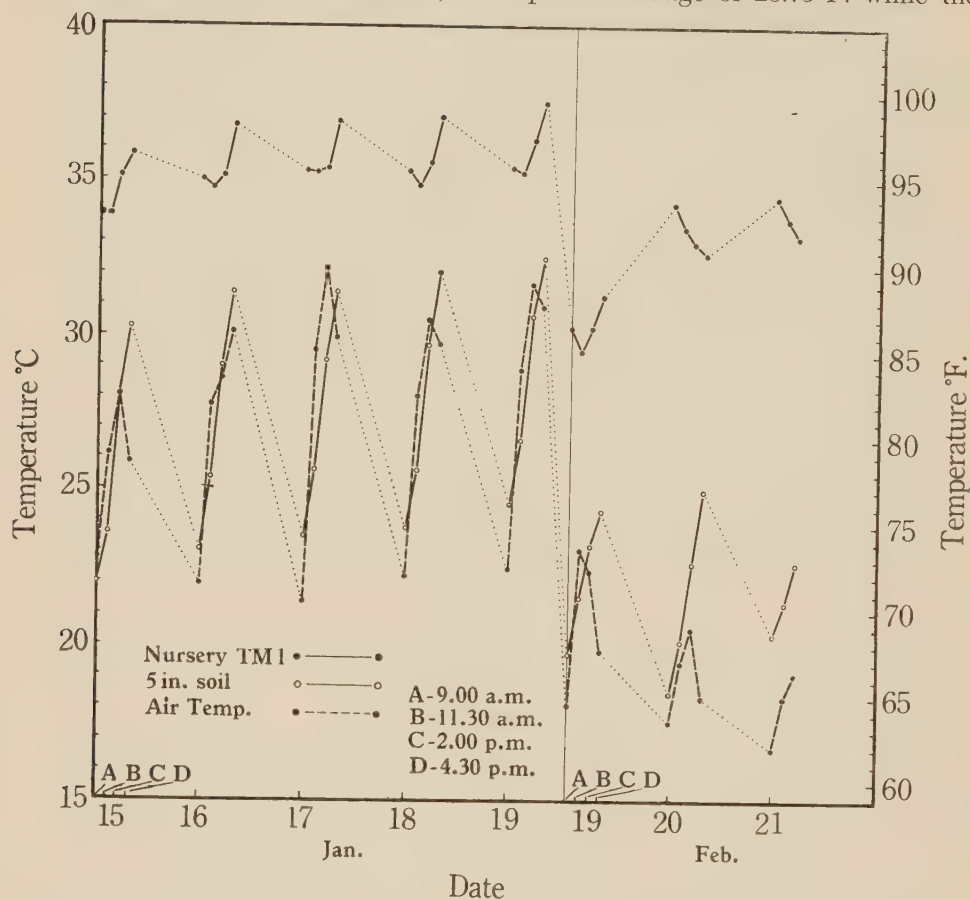


Fig. 11.—An initial drop in nursery temperature associated with a marked drop in environmental temperature at a season of the year when a comparatively small number of termites is present in the mound is shown. A continuation of low temperatures results in a rise in nursery temperature, following a return of termites to the mound. Readings taken on successive days are linked by dotted lines.

nursery of the unoccupied mound, at a point approximately in line with ground level, shows, over the same period, a range of only 3°F. One is therefore led to the conclusion that the reason probably lies in the structure and composition of the mound. The facts available at present are insufficient for a complete explanation, but it would appear that the nursery, because of its concentric, thin-walled construction which produces a large amount of air spaces and dead-end cavities, is a region of low thermal conductivity, in which major changes of temperature take place relatively slowly.

V. THE BUFFERING EFFECT OF TERMITE MOVEMENTS ON MOUND TEMPERATURES

Evidence has been secured which suggests that the presence of termites in the mound, or more probably the movement of termites into the mound, is capable of a buffering effect on mound temperatures against a sudden drop in environmental temperature. Figure 10 shows the nursery temperature of TM1 and the temperature of the environment during October, when there would still be a comparatively large number of termites present in the mound. During the second week there was a marked drop in the air temperature but the corresponding fall in nursery temperature was not very marked. Figure 11 shows similar records obtained in January and February, when the number of termites present in the mound would be comparatively low. The drop in temperature between the middle of January and the middle of February was markedly reflected in a decrease in nursery temperature. However, with a further drop in air temperature, the nursery temperature rose, the rise presumably being due to a return of termites to the mound.

In a series of laboratory experiments extending over a year, no definite preferred temperature could be demonstrated for *E. exitiosus*. On a given date, a given aggregation of termites from a mound distributed itself over a range of temperatures in such a way as to form a distribution polygon. While there might have been an accumulation of termites over a limited range which could be called the "range of preferred temperature," some termites always occurred at the extremes of a comparatively long range. It is suggested that the individuals comprising such aggregations are respectively in different physiological conditions characterized by different temperature tolerances. If this suggestion is correct, the explanation of the movement of termites to and from the mound will probably be obtained through an understanding of the effect of changes in mound and soil temperatures upon individuals with particular temperature tolerances.

Our observations give little evidence that termites are capable of lowering mound temperatures when environmental temperatures are high, other than by departure of termites from the mound. Early in the study it seemed possible that, during the summer, mound temperatures may be kept at a reduced level by the evaporation of moisture produced within the mound by the metabolism of the termites. If there is any lowering of mound temperature by this means it is apparently insignificant.

Small circular holes were observed in both TM1 and TM2 on October 24, 1933, the day after the emergence of alates from TM2. The hole in TM2 was found on the morning of October 24. The previous day there had been considerable activity within the mound and the temperature had reached 92.9°F. at 4.30 p.m. The hole in TM1 was made between 9.00 a.m. and 11.30 a.m. when the nursery temperature had risen to at least 93.4°F. The hole had been closed by 2.00 p.m. when the nursery temperature was still 93.4°F. although by 4.30 p.m. it had fallen to 92.1°F. On at least one occasion before this observation, and on numerous occasions after, the nursery temperature was higher than 93.4°F. and on none of them was a hole observed. Rather than for reducing temperature,

it seems that the hole was for the purpose of reducing carbon dioxide concentration which would have increased to an excessive amount following a period of high temperature when a large proportion of the colony was still within the mound. Ventilation holes in termitaria and underground nests have been referred to by a number of workers (see particularly Petch 1906; Hegg 1922; Emerson 1937; and Grassé 1937), while in *E. exitiosus* vertical galleries extend from the top of the nursery up towards the summit of the mound.

The temperature of poikilothermic animals is approximately the same as that of the medium in which they occur. However, there is evidence to show that their temperature may not be identical with that of the environment. The temperature of individual termites is apparently slightly above that of their surroundings, and, by virtue of large numbers massed together in the thin-walled nursery, the temperature level may be raised as much as 18.6°F. above the temperature which would occur in the mound were the termites not present.

Thus it is apparent that the statement that termites maintain their nests at a constant temperature does not apply to *Eutermes exitiosus*. It would appear that, by virtue of its shape and structure, a mound of *E. exitiosus* is comparatively constant in temperature over a limited period, provided the daily fluctuations in air temperature are of a normal magnitude. If, however, marked fluctuations occur, the mound temperature may be affected thereby, the magnitude of the effect being influenced by the buffering action of termites present in the mound initially or returning to the mound from outside galleries.

VI. OBSERVATIONS ON THE RELATION BETWEEN NYMPHS OR ALATES AND MOUND TEMPERATURE

The observations on TM2 discussed above suggested that mound temperature was affected by the presence in the mound of particular castes, notably nymphs or alates. Several pairs of mounds were therefore selected for similarity of size, habitat, aspect, and slope of habitat. The temperatures of the respective pairs were recorded and then the mounds were examined to determine which castes were present. The particulars obtained from these mounds are given in Table 3. Only two of the eleven pairs listed showed marked differences in temperature; in both pairs there was a difference in the composition of the colonies; the mound with the higher temperature contained a large number of alates, that with the lower temperature had none.

Two points emerge from these data:

- (1) Mounds of similar size in similar habitats have similar temperatures if the composition of the colonies is similar.
- (2) The presence of a large number of alates in the mound results in a higher temperature within the mound than would occur were alates not present.

VII. PRACTICAL APPLICATIONS OF THE OBSERVATIONS ON MOUND TEMPERATURE

Large numbers of *Eutermes exitiosus* are used in laboratory studies of resistance of materials to termite attack, 5,000 worker termites and the soldiers

TABLE 3

THE CASTES PRESENT AND THE TEMPERATURE OF PAIRS OF COMPARABLE MOUNDS

Mound No.	Habitat	Aspect	Size of Mound		Date	Time of Temp. Reading (a.m.)	Temp. (°F.)	Difference between Temp. of the Two Mounds in Each Pair (°F.)	Castes present in addition to Workers and Soldiers: + = present; - = absent			Remarks
			Horizontal Dimensions at Ground Level	Height (in.)					Eggs	Young	Juveniles	
1	Open grass-land	NNW.	2 ft. 10 in. x 2 ft. 10 in.	12	13.x.33	10.30	77.5	13.5	+	+	+	Nymphs concentrated at bottom
2	"	NNW.	3 ft. 0 in. x 3 ft. 0 in.	11	"	10.45	91.0		+	+	Few only	Alates all through inner wall and nursery
3	Open roadside	NNW.	2 ft. 0 in. x 2 ft. 2½ in.	11	19.x.33	10.00	87.2	0.6	-	-	+	Termites concentrated at bottom in both mounds
4	"	N.	2 ft. 3 in. x 2 ft. 4 in.	9½	"	9.45	87.8		-	-	+	
5	Forest	NW.	2 ft. 3 in. x 2 ft. 5½ in.	10	20.x.33	9.45	84.3	3.7	Few	-	-	
6	"	NW.	2 ft. 6 in. x 2 ft. 8 in.	9	"	9.30	88.0		-	-	-	
7	Forest area	NNW.	3 ft. 2 in. x 3 ft. 8 in.	14	23.x.33	9.45	93.8	3.2	Many	+	+	Alates fully pigmented in both mounds
8	"	NNW.	3 ft. 0 in. x 3 ft. 0 in.	13	"	10.00	98.0		Many	+	+	
9	Cleared land	WSW.	3 ft. 1 in. x 3 ft. 5 in.	11	27.x.33	10.35	92.0	3.0	+	+	+	
10	"	WNW.	3 ft. 2 in. x 3 ft. 4 in.	12	"	10.25	95.0		+	+	+	
11	Cleared land	W.	3 ft. 1 in. x 3 ft. 3 in.	17	27.x.33	10.55	92.4	9.7	+	+	+	Many
12	"	W.	3 ft. 1 in. x 3 ft. 1 in.	15	"	10.45	81.7		+	+	+	
13	Cleared land	NNE.	3 ft. 3 in. x 3 ft. 8 in.	15	26.x.33	10.00	96.5	0.6	-	-	+	Alates concentrated about and below ground level in both mounds
14	"	NW.	3 ft. 7 in. x 3 ft. 8 in.	16	"	10.15	95.9		-	-	+	
15	Cleared land	NNW.	2 ft. 10 in. x 2 ft. 10 in.	10	26.x.33	11.15	93.0	0.4	+	+	+	
16	"	NW.	3 ft. 0 in. x 3 ft. 4 in.	11½	"	11.00	93.4		+	+	+	
17	Cleared land	NW.	3 ft. 4 in. x 3 ft. 7 in.	16	27.x.33	10.10	93.4	1.4	+	+	+	Few only
18	"	NW.	3 ft. 5 in. x 3 ft. 11 in.	17	"	10.00	94.8		+	+	+	
19	Open grass-land	W.	3 ft. 0 in. x 3 ft. 1 in.	17	10.xi.33	11.20	89.6	1.1	Many	+	+	Alates in both mounds flew readily when mound was opened
20	"	W.	3 ft. 2 in. x 2 ft. 11 in.	14	"	11.12	90.7		Many	+	+	
21	Open grass-land	W.	2 ft. 10 in. x 2 ft. 10 in.	14	10.xi.33	11.15	90.0	1.7	Many	+	+	Alates concentrated at bottom of both mounds
22	"	W.	3 ft. 0 in. x 3 ft. 5 in.	15	"	11.07	88.3		Many	+	+	

• The term "juveniles" is used to describe immature non-productive forms, whilst the term "nymphs" signifies immature reproductive forms.

accompanying them being used for each standard laboratory colony (Holdaway 1935). Usually from twenty to thirty colonies are set up at a time, so that from 100,000 to 200,000 healthy termites are required on each occasion. In securing the termites from the mound material a large number is damaged. In order to allow a margin of safety in the number of sound termites, and to allow for loss through damage, mound colonies are needed of much greater numerical strength than the number of termites actually required. At any particular time of the year there is a limited range of temperature characteristic of vital populous mounds. By reading the temperature of the nursery of mounds before they are dug up, and ignoring such mounds as do not give a sufficiently high temperature, one can be reasonably sure of securing requirements. This procedure is particularly valuable in the warmer months when large numbers of termites are away from the mound, and a random choice of mounds would involve greater risk of there being insufficient termites for requirements. Figure 12 gives the nursery temperature of 170 mounds which have been used for laboratory and field testing over the past few years. This figure shows the seasonal march of mound temperatures which has already been demonstrated above.

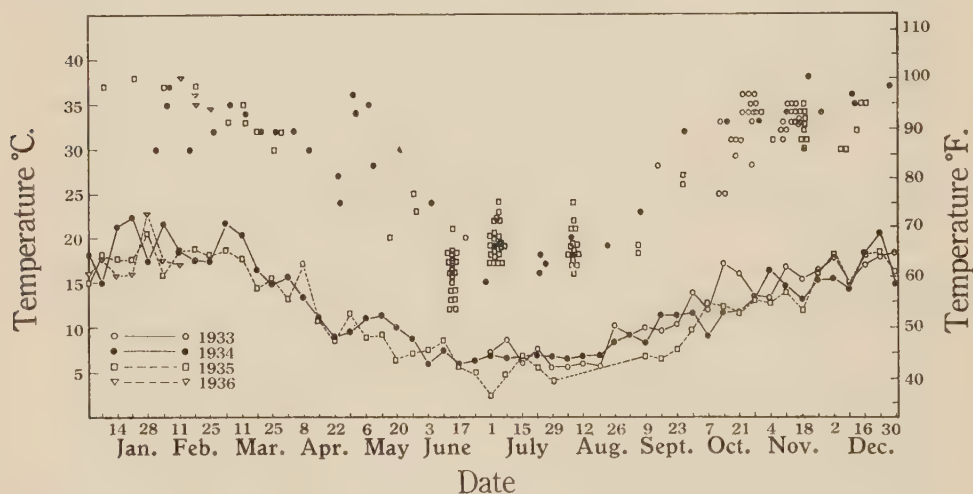


Fig. 12.—The nursery temperatures of 170 mounds, taken during the period June 1933 to February 1936, inclusive, plotted with the mean weekly air temperatures over the same period. The scatter in mound temperatures, on or about any particular date, is due largely to the fact that the mounds were in a variety of situations, and although of similar general dimensions, were not absolutely of the same size. Air temperatures in 1933 and 1934 are linked by continuous lines and those of 1935 and 1936 by broken lines.

Mound temperature records also serve as a valuable check on the vitality of mounds used for field tests of resistance of materials to termite attack. A highly satisfactory method of evaluating resistance of materials and treatments has now been developed. By means of mound temperature records, the use of only healthy, vital colonies for the tests is assured. This is particularly important in tests which may continue for many years. Moreover, by eliminating vitality

of colonies from the category of uncontrollable variables, the significance of other variables can be more readily observed, and a more satisfactory determination of the resistance of the material under test be secured. Further, by having temperature tubes permanently installed in the nursery of the mounds and recording the temperature at intervals during the course of tests, a check on the vitality of the respective colonies can be kept throughout the test, ensuring that reduction in vitality or death of a colony will not pass unobserved for a lengthy period. One example will serve to illustrate the value of mound temperatures in this connection.

In the winter of 1936, field tests of treated and untreated timbers involving 1,030 samples and 38 mounds of *Eutermes exitiosus* were begun. According to the records of nursery temperatures all mounds contained populous colonies at the beginning of the tests which were initiated during August. In December, inspection of the exterior of the test mounds suggested that three of the mounds were reduced in vitality. Nursery temperatures of the three mounds and of other test mounds in the immediate vicinity were read. These temperature records indicated that two of the three mounds were probably dead but that the third was satisfactory. These readings taken on December 9, 1936, with an average air temperature of 22.2°C., were as follows: externally healthy mounds, 33.4°, 33.6°, 31.4°, 33.0°, 34.1°, 33.3°, 36.2°C.—average 33.7°C.; mounds of doubtful vitality, 34.9°, 21.0°, 20.5°C. In this latter group, the mound with a nursery temperature of 34.9°C., although suspected of reduced vitality, was found to be satisfactory, while the remaining two mounds with nursery temperatures of 21.0° and 20.5°C., showed on examination that the colonies associated with them were dead.

As a result of the observations just cited it was decided to read the nursery temperatures of all the mounds being used for test purposes. These mounds are divided between two test sites which are in similar country and only three to four miles apart across country. Recording was begun at the two sites simultaneously. On December 23, 1936, all records were completed in just over two hours, during which time the air temperature ranged from 22.0° to 24.4°C. The readings at both sites were very similar and so records from only one site are given here. Eighteen mounds had nursery temperatures ranging from 31.2° to 38.0°C. and averaging 34.1°C., while one mound had a nursery temperature of 24.4°C. These records showed that the colony associated with this latter mound was apparently dead, a fact which had not been suspected from the superficial routine inspection carried out a few weeks previously.

The reason for the death of these colonies need not be referred to in detail here; it apparently lay in certain of the materials being tested. As a result of the death of the colonies an aspect of field testing not formerly suspected was brought to light. The main point in citing the records is to illustrate the fact that mound temperatures give a more reliable indication of the vitality of colonies than does a superficial examination of the mounds; moreover, if the vitality of the colonies initially had not been without question, it is doubtful whether the significance of the death of the colonies would have been indicated.

A third practical aspect of this study is its application in testing insecticides for direct control of mound colonies of termites. For positive proof of the efficiency of any insecticides or for accurate comparison of the relative toxicities of different insecticides to termites, it is essential that mounds selected for such work be occupied by normal healthy colonies. This information can be rapidly obtained under field conditions by inserting a long-stemmed thermometer into an auger hole bored to the central region of the mound and thus recording the nursery temperature. Simultaneously, a reading of the air temperature in the shade is taken and the condition of the colony can be gauged satisfactorily from a comparison of these two readings. Whenever these readings indicate the presence of a normal healthy colony, the auger hole is then used as a most efficient channel for conveying the insecticide to the centre of the mound where the termite population tends to be most concentrated.

This method of demonstrating the original healthy condition of mound colonies of termites used for insecticidal studies has now been in use for some time and it has enabled the relative efficiencies of a large number of toxic materials to be determined accurately.

VIII. ACKNOWLEDGMENTS

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IX. REFERENCES

- ANDREW, B. J. (1930).—Method and rate of Protozoan refaunation in the termite *Termopsis angusticollis* Hagen. *Univ. Calif. Publ. Zool.* **33** (21): 449-70.
- ANDREWS, E. A. (1927).—Ant mounds as to temperature and sunshine. *J. Morph.* **44** (1): 1-20.
- COWLES, R. B. (1930).—The life history of *Varanus niloticus* (Linnaeus) as observed in Natal, South Africa. *J. Ent. Zool.* **22** (1): 1-31.
- EMERSON, A. E. (1937).—Termite architecture. *Nat. Hist.* **39** (4): 241-8.
- FYFE, R. V., and GAY, F. J. (1938).—The humidity of the atmosphere and the moisture conditions within mounds of *Eutermes exitiosus* Hill. Coun. Sci. Industr. Res. Aust., Pamph. No. 82, 22 pp.
- GATES, B. N. (1914).—The temperature of the bee colony. U.S. Dep. Agric., Bull. No. 96, 29 pp.
- GRASSÉ, P. P. (1937).—Le *Bellicositermes jeanneli* n. sp. constructeur de grandes termitières à cheminée. *Bull. Soc. Ent. Fr.* **42** (5): 71-3.
- HEGH, E. (1922).—"Les Termites." 756 pp. (Bruxelles.)
- HILL, G. F. (1930).—White ant investigations in the Federal Capital Territory. *J. Coun. Sci. Industr. Res. Aust.* **3**: 220-4.
- HILL, G. F. (1932).—Termites (white ants) in south-eastern Australia. Coun. Sci. Industr. Res. Aust., Pamph. No. 25, 27 pp.
- HIMMER, A. VON (1932).—Die Temperaturverhältnisse bei den sozialen Hymenopteren. *Biol. Rev.* **7** (3): 224-53.

- HOLDAWAY, F. G. (1933).—The composition of different regions of mounds of *Eutermes exitiosus* Hill. *J. Coun. Sci. Industr. Res. Aust.* **6**: 160-5.
- HOLDAWAY, F. G. (1935).—Standard laboratory colonies of *Eutermes exitiosus* Hill for testing timber under controlled conditions. *Aust. Inst. Agric. Sci.* **1** (1): 34-5.
- HOLDAWAY, F. G., GAY, F. J., and GREAVES, T. (1935).—The termite population of a mound colony of *Eutermes exitiosus* Hill. *J. Coun. Sci. Industr. Res. Aust.* **8** (1): 42-6.
- HOLDAWAY, F. G., and HILL, G. F. (1936).—The control of mound colonies of *Eutermes exitiosus* Hill. *Ibid.* **9** (2): 135-6.
- PETCH, T. (1906).—The fungi of certain termite nests (*Termes redemanni*, Wasm., and *Termes obscuriceps*, Wasm.). *Ann. R. Bot. Gdns., Peradeniya* **3**: 185-270.
- PHILLIPS, E. F., and DEMUTH, G. S. (1914).—The temperature of the honey bee cluster in winter. U.S. Dep. Agric., Bull. No. 93, 16 pp.
- SNYDER, T. E. (1929).—Friends and foes of termites or white ants. *Zool. Anz.* **82**: 40-6.
- STEINER, A. (1930).—Neuere Ergebnisse über den sozialen Wärmehaushalt der einheimischen Hautflügler. *Naturwissenschaften* **18**: 595-600.
- ZANDER, E. (1917).—Die Temperaturverhältnisse im Bienenstock während des Winters. *Z. Angew. Entom.* **4**: 24-30.

EXPLANATION OF PLATE 1

Mound TM1 with temperature tubes in position and meteorological screen near by. Soil tubes are protected with a small wooden guard on the south side.



HOLDAWAY and GAY.—TEMPERATURE STUDIES OF THE HABITAT OF *EUTERMES EXITIOSUS*

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